UNIVERSIDADE FEDERAL DO PARANÁ

ALINE CRISTINA MARTINS

Evolução das abelhas coletoras de óleos florais *Centris* e *Epicharis*: inferências a partir da filogenia molecular datada da subfamília Apinae e das plantas produtoras de óleo floral da Região Neotropical

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Tese apresentada como requisito parcial à obtenção do grau de Doutor em Ciências Biológicas, Área de concentração Entomologia, no Curso de Pós-Graduação em Entomologia, Setor de Ciências Biológicas, da Universidade Federal do Paraná

Orientador: Prof. Dr. Gabriel A. Rodrigues de Melo
Co-orientadora: Prof. Dr. Susanne S. Renner

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Tese aprovada como requisito parcial para obtenção do grau de "Doutor em Ciências", no Programa de Pós-graduação em Ciências Biológicas, Área de Concentração em Entomologia, da Universidade Federal do Paraná, pela Comissão formada pelos professores:

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“hoje (...) estou mais casmurro do que nunca, sou metade da pessoa que fui em miúdo, valho menos, muito menos. mas quero nunca esquecer o quanto esperei do mundo, o quanto esperei da arte, da amizade, do país. espero menos, cada vez menos, por isso me importa lembrar, para ser sempre capaz de não desistir. porque quero nunca desistir. completar um aniversário serve para isto: medirmos a que distância estamos de ver o mundo melhor. e serve para parabenizarmos todos quantos acompanham a mesma esperança que temos nós e, de igual modo, não desistem. não desistir é sempre uma forma de sonhar. e envelhecer só vale a pena por esse motivo: por estarem aí, algures, a sonhar com essa maravilha de sermos gente melhor. obrigado.

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SUMÁRIO

RESUMO

As abelhas são insetos dependentes das angiospermas, com as quais compartilham uma história de cerca de 120 Ma. Desde sua origem, a relação entre abelhas e flores evoluiu em uma mistura de interações obrigatórias e facultativas, que resultaram em adaptações fantásticas em ambas as partes. Pólen e néctar são as recompensas comumente ofertadas pelas plantas a polinizadores. Porém, cerca de 2000 espécies de angiospermas produzem óleo em suas flores, que é coletado por cerca de 450 espécies de abelhas especializadas e dependentes deste recurso para alimentação de suas larvas e nidificação. Na Região Neotropical, esse intrigante mutualismo é protagonizado pela diversa família Malpighiaceae e pelas cerca de 250 espécies de abelhas dos gêneros *Centris* e *Epicharis*. Estas abelhas são formalmente classificadas em uma tribo, Centridini, agrupamento corroborado pelas filogenias morfológicas devido ao suporte dado pelos caracteres relacionados à coleta do óleo floral. *Centris* e *Epicharis* se destacam na fauna de abelhas Neotropical devido a riqueza de espécies, ao tamanho de seus indivíduos, ampla distribuição e interações com diversas espécies de plantas, incluindo algumas de importância socioeconômica. A recente contribuição das filogenias moleculares indicou a parafilia de Centridini, porém com baixa amostragem nestes dois gêneros. Na presente tese a monofilia de Centridini foi investigada, bem como suas relações internas, através de uma robusta filogenia molecular baseada em 2/3 de suas espécies e uma ampla amostragem de grupo-externo, totalizando 174 espécies de Apinae e mais de 4000 nucleotídeos alinhados (Capítulos 1 e 2). A datação através de relógio molecular baseou-se em dois pontos fósseis de calibração, *Apis lithohermea*, do Mioceno do Japão e *Kelneriapis eocenica*, do âmbar do Báltico. Além disso o presente trabalho procurou elucidar a evolução de *Centris* e *Epicharis* em associação com plantas produtoras de óleo floral da Região Neotropical (Capítulos 3 e 4). Para tanto também foram produzidas filogenias moleculares datadas, baseadas em dados inéditos ou provenientes do GenBank, para as plantas que são fontes de óleo floral destas abelhas: Calceolariaceae, Krameriaceae, Iridoidea (Iridaceae) e *Nierembergia* (Solanaceae). A segunda fonte de óleo mais importante para *Centris* depois de Malpighiaceae, a tribo Angelonieae (Plantaginaceae), foi pela primeira vez aqui investigada sob uma perspectiva filogenética, associada a datação e biogeografia, para entender a evolução da associação com *Centris* (Capítulo 4). A filogenia da subfamília Apinae corroborou a parafilia de Centridini, sendo *Centris* grupo-irmão das abelhas corbiculadas e *Epicharis* irmão de ambos, formando a linhagem Apine. Esta linhagem provavelmente originou-se no Novo Mundo, no Cretáceo

Inferior, há 91 Ma. A coleta de óleo floral é ancestral neste grupo, foi perdida nas abelhas corbiculadas e em algumas linhagens de *Centris.* A filogenia de *Centris* e *Epicharis* rejeita parcialmente a atual classificação taxonômica, sendo aqui propostos alguns rearranjos, principalmente para as espécies atualmente classificadas em *Centris* (*Paracentris).* As futuras proposições de uma nova classificação baseadas na presente filogenia molecular, entretanto, requerem ainda um estudo de caracteres morfológicos que facilitem o reconhecimento dos novos táxons propostos. Enquanto a literatura indica que Malpighiaceae se originou no Cretáceo, iniciando o mutualismo com as abelhas coletoras de óleo, os demais grupos aqui datados, começaram a se originar apenas durante o Eoceno, com Krameriaceae e Angelonieae (Plantaginaceae). E no Mioceno, outras origens da produção de óleo floral ocorreram em Iridaceae, Solanaceae e Calceolariaceae. Enquanto *Epicharis* permaneceu associado exclusivamente com Mapighiaceae, *Centris* diversificou-se em áreas mais secas da América do Sul e do Norte e se associou com novas fontes de óleo, principalmente *Calceolaria*, Angelonieae e Krameriaceae. Ao menos quatro perdas do hábito coletor de óleo ocorreu em *Centris* em áreas xéricas, onde as fontes de óleo floral são escassas. No capítulo 4, observamos que estas áreas secas também foram importantes na evolução de Angeloniae, local onde provavelmente originaram-se no Eoceno Médio, simultaneamente com seus principais polinizadores do gênero *Centris.* A produção de óleo no grupo originou-se quatro ou cinco vezes nos últimos 25 Ma. Os resultados deste quarto capítulo são peças-chave no entendimento do nicho evolutivo das abelhas coletoras de óleo, mas também revela importantes informações sobre a sistemática e biogeografia das Plantaginaceae neotropicais.

ABSTRACT

Bees are insects that feed on angiosperms and share with these plants a 120 Ma history of evolution. Since its origin, the relationship between bees and flowers evolved through a mix of obligatory and facultative dependencies, which originated amazing adaptations in both partners. Pollen and nectar are the most common reward offered by plants to pollinators. However, about 2000 species of angiosperms produce floral oils, collected by circa 450 species of specialized bees, which rely on this resource for larval feeding and nest construction. In the Neotropical Region, the diverse family Malpighiaceae and about 250 species in the bee genera *Centris* and *Epicharis* are protagonists in this intriguing mutualism. Those bees, *Centris* and *Epicharis*, are formally classified in one tribe, Centridini, grouping corroborated by morphological phylogenies due to the support given by the characters adapted to the oil collecting. *Centris* and *Epicharis* are important components of Neotropical bee fauna due to the richness, big individual size, wide distribution and interactions with several species of plants, including some of socioeconomic importance. The recent contribution of molecular phylogenies indicated the paraphyly of Centridini, however, with a very low sampling. In the present thesis, the monophyly of Centridini and its inner relationships were investigated through a robust molecular phylogeny based on two thirds of the Centridini species and a wide sampling of outgroups, totalizing 174 species in Apinae and more than 4000 aligned nucleotides (Chapters 1 and 2). The molecular clock dated phylogeny was based in two fossil calibration points, *Apis lithohermea*, from the Miocene of Japan, and *Kelneriapis eocenica*, from the Baltic amber. Besides, the present study addressed the evolution of *Centris* and *Epicharis* in association with the Neotropical oil producing plants (Chapters 3 and 4). Therefore molecular-clock dated phylogeny were produced, based on newly produced or GenBank data, for all oil host plants of these bees: Calceolariaceae, Krameriaceae, Iridoidea (Iridaceae) and *Nierembergia* (Solanaceae). The second most important oil source for *Centris* after Malpighiaceae, the tribe Angelonieae (Plantaginaceae), was for the first time investigated on a phylogenetic approach, associated with dating and biogeography, for understanding the evolution with *Centris* (Chapter 4). The phylogeny of the subfamily Apinae corroborated the paraphyly of Centridini, being *Centris* sister-group of corbiculate bees and *Epicharis* sister to both, forming the Apine line. This lineage probably originated in the New World, in the Early Creataceous, 91 Ma ago. The floral oil collecting is ancestral in this group and it was lost in the corbiculate bees and in some lineages of *Centris*. The phylogeny of *Centris* and *Epicharis* partially rejects the present taxonomic classification,

thus some taxonomic rearrangements are here proposed, mainly for the species classified in *Centris* (*Paracentris).* The future propositions of a new classification based on the molecular phylogeny, however, require the deeper study of morphological characters for the recognition of newly proposed taxons. The literature indicates a cretaceous origin of Malpighiaceae and probable start of mutualism with oil collecting bees, however the remaining oil producing groups here dated began to originate only in the Eocene, being Krameriaceae and Angeloniae (Plantaginaceae) the first. In the Miocene, other origins of floral oil production occurred in Iridaceae, Solanaceae e Calceolariaceae. While *Epicharis* remains exclusively associated to Malpighiaceae flowers, *Centris* diversified in the driest parts of South and North America and associated with new oil sources, mainly *Calceolaria,* Angelonieae and Krameriaceae. At least four losses of oil collecting behavior occurred in *Centris* in xeric regions, where the oil sources are scarce. In the Chapter 4, we observe the importance of those dry areas in the evolution of Angelonieae, where they probably originated in the Middle Eocene, simultaneously with their main *Centris* poll inators. The floral oil production originated four or five times in the last 25 Ma in Angelonieae. The results of the fourth chapter are key to understand the evolutionary niche of these oil-collecting bees, but also reveal important information on the systematics and biogeography of Neotropical Plantaginaceae.

INTRODUÇÃO GERAL

As abelhas são um grupo monofilético que surgiu há 100 a 120 milhões de anos, no Cretáceo, e que dependem somente dos recursos das angiospermas para alimentação (Michener 2007; Cardinal & Danforth 2013). Estes insetos são os grandes polinizadores das plantas com flores, sendo que suas fêmeas possuem várias adaptações morfológicas, comportamentais e fisiológicas que permitem a elas se alimentar nas flores, coletar recursos e carregar estes das flores para o ninho (Thorp 1979). Comumente, as abelhas coletam néctar para alimentar a si mesmas e pólen para as larvas, mas elas também podem coletar outros recursos florais, como resinas, perfumes e lipídios (Wcislo & Cane 1996).

Devido ao serviço ecológico realizado pelas abelhas na polinização de grande parte das cerca de 250 mil espécies selvagens e cultivadas de plantas floríferas, elas estão entre os grupos animais mais importantes economicamente (Kremen et al. 2002; Klein et al. 2007). Entretanto, este serviço ecológico está em risco devido a declínios documentados nas populações de abelhas ao redor do mundo (Biesmeijer et al. 2006; Bartomeus et al. 2013; Martins et al. 2013b). As maiores ameaças as populações nativas de abelhas são a perda de hábitat, competição com espécies exóticas, pesticidas e mudanças climáticas (Thomson 2004; Whitehorn et al. 2012; Tylianakis 2013).

As mais conhecidas espécies de abelhas – as melíferas do gênero *Apis* (*honeybees*) e mamangavas sociais do gênero *Bombus* (*bumblebees*) – são insetos altamente sociais, também chamados eussociais. Porém, a maior parte das espécies de abelhas possuem outros níveis de socialidade que vai do solitário ao semisocial (Michener 2007). Independentemente do seu comportamento social, as abelhas em geral constroem seus ninhos no solo ou em cavidades pré-existentes usando o próprio solo ou madeira como substrato, agregando diferentes materiais na impermeabilização do ninho e aprovisionamento das larvas (Michener 2007). Por outro lado, os cleptoparasitas são abelhas que não constroem seus próprios ninhos, ao invés disso, as fêmeas ovipõem nas células construídas por fêmeas de outras espécies e a larva cleptoparasita se alimenta do alimento aprovisionado pela fêmea hospedeira (Wcislo & Cane 1996). Este comportamento cleptoparasita é encontrado em 28% das espécies de Apidae, mas também ocorre nas famílias Halictidae e Megachilidae (Michener 2007; Cardinal et al. 2010).

As abelhas são divididas em dois grupos informais: abelhas de língua longa e abelhas de língua curta, que juntas compreendem sete famílias dentro de Apoidea (Hymenoptera). Estas famílias são Megachilidae e Apidae (língua longa), Stenotritidae, Colletidae, Andrenidae, Melittidae e Halicitidae (língua curta) (Michener 2007). Alternativamente, estes insetos

podem ser tratados como uma única família – Apidae – com sete subfamílias que correspondem as famílias da classificação tradicional acima citada, sendo mais consistente com aquela adotada para outras ordens de himenópteros aculeados (Melo & Goncalves 2005). Filogenias morfológicas estão disponíveis para ambos grupos, abelhas de língua longa e curta, em Roig-Alsina & Michener (1993) e Alexander & Michener (1995), respectivamente. Estudos filogenéticos recentes reavaliaram as relações entre os grandes grupos de abelhas mostrando que as abelhas de língua longa são de fato clado irmão das abelhas de língua curta, cujo membro mais basal é Melitinae (Danforth et al. 2006). Estes resultados contradizem a visão tradicional de que Colletidae seria a família de abelhas mais basal.

Nos últimos dez anos, nosso conhecimento sobre as relações filogenéticas entre as abelhas tem progredido muito (Danforth et al. 2013). Relacionamentos baseados em sequencias de DNA são agora conhecidos para grandes grupos (Danforth et al. 2004; Almeida & Danforth 2009; Cardinal et al. 2010; Litman et al. 2011) e para algumas tribos, como os Meliponini (Rasmussen & Cameron 2010), Euglossini (Ramírez et al. 2010), Ctenoplectrini (Schaefer & Renner 2008) e Ceratinini (Dew et al. 2011). Os genes ribossomais 18S e 28S estão entre as regiões do genoma das abelhas mais comumente usadas para investigar relações mais antigas nas reconstruções filogenéticas. Todavia, parece ser mais vantajoso o exame de regiões codificadoras de proteínas nucleares, por que elas evoluem mais lentamente que os genes mitocondriais e são mais fáceis de alinhar que as regiões ribossomais (Danforth et al. 2013). A presente tese de doutorado foi beneficiada por esse recente progresso nos métodos moleculares para filogenéticas entre as abelhas e irá tratar da evolução de um intrigante grupo de abelhas coletoras de óleos florais.

Relações evolutivas entre abelhas e angiospermas

As relações entre abelhas e polinizadores tem sido um grande tópico no estudo da evolução das flores desde Darwin (Darwin 1862). O mutualismo entre plantas e polinizadores é um dos clássicos exemplos em que a evolução é mediada por interações biológicas e podem levar a coevolução (Thompson 1989; Anderson & Johnson 2008). A despeito da relevância da coevolução em diversas interações mutualísticas, nas relações planta-polinizador, o processo coevolutivo é raro, e muitas vezes improvável. Na verdade, os níveis de especialização no mutualismo entre plantas e polinizadores pode variar de um pra um até interações extremamente difusas (Morgan 2000).

O estudo das interações entre plantas e polinizadores são conduzidos levando em consideração diferentes linhas de desenvolvimento baseadas em características ecológicas, por exemplo, a distinção entre visitantes e polinizadores efetivos (Johnson & Steiner 2000). Porém, uma abordagem interessante seria integrar características ecológicas com informações histórico-evolutivas, que são principalmente baseadas nas filogenias e registro fóssil (Martins 2013). Evidências diretas das interações entre plantas e polinizadores - por exemplo, partes florais fossilizadas junto ao corpo do polinizador, por exemplo (Ramírez et al. 2007) - são raras. Por outro lado, evidencias indiretas, como características morfológicas adaptadas as flores, são mais comuns (Grimaldi 1999). Este fato dificulta o entendimento das origens evolutivas das abelhas e nos dá apenas algumas pistas sobre as suas relações com as angiospermas. *Cretotrigona prisca* do Cretáceo Superior, com aproximadamente 80 milhões de anos, é considerado o fóssil de abelha mais antigo (Michener & Grimaldi 1988; Engel 2000) e é membro de um grupo altamente derivado, as abelhas indígenas sem ferrão (Meliponini). Apesar de controverso, o descobrimento de *Melittosphex burmensis*, um fóssil de 100 Ma, apresenta características de abelhas, poderia ser o mais antigo fóssil desse grupo (Poinar & Danforth 2006).

Na ausência do registro fóssil, análise filogenética é o caminho mais lógico para traçar as origens das adaptações morfológicas, comportamentos específicos e relações mutualísticas entre abelhas e plantas. O uso de uma abordagem filogenética e comparativa tem sido a mudança mais notável no estudo das interações entre plantas e polinizadores, especialmente através das análises derivadas das filogenias moleculares (Renner & Schaefer 2010; Smith 2010; Ramírez et al. 2011; Chartier et al. 2013).

Análises filogenéticas podem levar ao entendimento da evolução de diversas características que podem ser mapeadas nas filogenias: morfológicas (Armbruster 1997); trocas de polinizadores (Cosacov et al. 2009; Chauveau et al. 2011) ou trocas de plantas hospedeiras (Larkin et al. 2008; Michez et al. 2008). Mesmo para grupos de abelhas especializadas (e.g. Rophitinae), trocas de plantas hospedeiras, quando mapeadas nas filogenias mostram um grande número de duplicações e ausência de co-cladogênese, indicando pouca correlação entre abelhas e a filogenia das plantas (Patiny et al. 2008). Trocas de plantas hospedeiras é, ao contrário, comum na evolução das abelhas, e é afetada mais pela disponibilidade de recursos do que pela proximidade filogenética das plantas hospedeiras (Sipes & Tepedino 2005; Michez et al. 2008).

Oligoleticia aparece em alguns clados mais basais de abelhas como uma condição ancestral que evoluiu em direção a polileticia (Larkin et al. 2008; Michez et al. 2008; Renner

& Schaefer 2010; Litman et al. 2011). Fortes evidências de trocas de hospedeiros e aumento da variedade de hospedeiros visitados, assim como a baixa congruência entre as filogenias de abelhas e plantas, reforça a ideia das relações entre plantas e polinizadores como uma mistura de dependências obrigatórias e facultativas. A despeito de esforços em conceituar as relações entre plantas e polinizadores de acordo com categorias de especialização (e.g. síndromes florais; Faegri & van der Pijl 1966), este tipo de mutualismo é dinâmico e generalista (Waser et al. 1996), mesmo que alguns grupos funcionais possam ser observados (Fenster et al. 2004).

Óleo como recurso floral

Comumente abelhas em visita às flores coletam néctar e/ou pólen e, dependendo do tamanho, comportamento e outras características, podem atuar como polinizadores efetivos. Além das recompensas mais comuns, as flores também podem ofertar recursos menos usuais, como óleos, resinas e perfumes, a polinizadores especializados (Simpson & Neff 1981). Os mais comuns são os óleos produzidos por cerca de 2000 espécies em 11 famílias de angiospermas e coletados pelas fêmeas e, em alguns casos machos, de cerca de 447 espécies de abelhas nos gêneros *Centris* (230 espécies), *Epicharis* (31), *Tetrapedia* (28), *Ctenoplectra* (19), *Macropis* (16), *Rediviva* (24) e 12 gêneros dentro de Tapinotaspidini (95) (Vogel 1974; Renner & Schaefer 2010; Cappellari et al. 2011).

A origem da produção de óleos florais é polifilética nas plantas e assume-se, através de filogenias moleculares e datação, que esta característica evoluiu ao menos 28 vezes e foi perdido 36-40 vezes (Renner & Schaefer 2010). As flores que produzem óleo têm morfologia diversa, mas todas tem em comum a presença de uma área denominada elaióforo, que pode ser epitelial – uma série de células epidérmicas secretoras de óleo cobertas por uma fina cutícula – ou tricomáticas – um campo de centenas ou milhares de pelos glandulares (Vogel 1974). Muitas revisões sobre a produção de óleos florais e seus visitantes, compilando toda literatura produzida desde sua descoberta, há 40 anos, têm sido produzidas (Vogel 1974; Neff & Simpson 1981; Simpson & Neff 1981; Buchmann 1987; Alves-dos-Santos et al. 2000; Cocucci et al. 2000; Rasmussen & Olesen 2000; Machado 2004), mas apenas Renner & Schaefer (2010) utilizaram uma abordagem filogenética em sua compilação.

Apenas abelhas coletam óleos florais, e este comportamento está restrito a aproximadamente 500 espécies (Vogel 1974; Michener 2007). Através de filogenias moleculares disponíveis atualmente, é possível inferir que o comportamento de coletar óleo

evoluiu ao menos seis vezes: 1. Centridini – se a classificação tradicional é considerada, teríamos um ganho, porém se Centridini é considerada parafilética (Cardinal et al. 2010), poderíamos ter dois ganhos, um em *Centris* e outro em *Epicharis*; 2. Tapinotaspidini; 3. Tetrapediini 4. *Ctenoplectra;* 5. *Macropis* e 6. *Rediviva*. Este comportamento foi perdido ao menos duas vezes em espécies não coletoras de *Centris* e no gênero *Ctenoplectrina* (Schaefer & Renner 2008; Renner & Schaefer 2010).

É bem conhecido o fato de que o comportamento de coleta de óleo é uma característica polifilética, mas a questão de quantas vezes essa característica surgiu ou foi perdida encontra-se vagamente respondida. A reconstrução de um completo cenário seria possível apenas com a filogenia dos grupos que contém espécies de abelhas coletoras de óleo. Atualmente, reconstruções filogenéticas estão disponíveis apenas para grupos do Velho Mundo; não foi publicada até o momento qualquer filogenia dos grupos Neotropicais. A despeito deste fato, muitas comparações podem ser feitas, apesar de serem limitadas devido à grande diversidade de espécies coletoras de óleo na Região Neotropical: Centridini (cerca de 250 espécies), Tapinotaspidini (cerca de 95 espécies) e *Tetrapedia* (28 espécies) (Moure et al. 2013).

A tribo Centridini

A tribo Centridini ocorre na região Neotropical e parte da região Neártica, distribuindo-se da Argentina até o sul dos Estados Unidos em diferentes tipos vegetacionais. Estes podem incluir desertos, florestas tropicais, savanas e caatinga. A tribo é composta por dois gêneros (*Centris* e *Epicharis*), 21 subgêneros, e aproximadamente 250 espécies (Moure et al. 2012). Elas são solitárias, de tamanho médio a grande, robustas, pilosas e voam muito rápido (Michener 2007). Grande parte das fêmeas constroem seus ninhos no solo, em barrancos ou no solo plano (Aguiar & Gaglianone 2003) e algumas delas usam cavidades pré-existentes em madeira morta para nidificação (Frankie et al. 1993). Centridini é o grupo com maior número de espécies coletoras de óleos florais, e é responsável por 73% de todas as visitadas registradas para as flores com óleo (Machado 2004). Elas usam óleo floral, em lugar de ou associado ao néctar, para alimentação das larvas, devido ao seu alto conteúdo calórico (Vinson et al. 1995). Elas também o usam para impermeabilização das células e para ajudar na coleta de outros materiais para nidificação, como areia e pedaços de madeira (Vinson et al. 1997; Gaglianone 2005). O néctar é coletado por machos e fêmeas para alimentação própria (Vinson et al. 1997).

Abelhas coletoras de óleo possuem diversas adaptações nas suas pernas anteriores e médias para coleta deste recurso (Neff and Simpson 1981). O padrão de aparatos coletores mais comum em Centridini consiste em um conjunto de cerdas morfologicamente diversas e um pente de cerdas rígidas primário e secundário nas quatro pernas (padrão *four-legged*) (Neff & Simpson 1981). Em *Epicharis*, todas as espécies apresentam esse padrão, mas alguns grupos de *Centris* apresentam modificações (Neff & Simpson 1981). Por exemplo, as espécies de *Centris* do grupo *hyptidis* (Vivallo & Melo 2009), o subgênero *Wagenknechtia* (Simpson et al. 1990), e algumas espécies norte-americanas de *Paracentris* perderam completamente os aparatos coletores e não coletam óleos florais (Neff & Simpson 1981).

A importância de *Centris* e *Epicharis* na fauna de abelhas Neotropicais deve-se ao grande número de espécies, grande tamanho de seus indivíduos, ampla distribuição e interações com diversas espécies de plantas, incluindo algumas de importância socioeconômica, como a castanha-do-pará, (Lecythidaceae: Maués 2002), a aceroleira (Malpighiaceae: Oliveira & Schlindwein 2009) e os maracujás (Passifloraceae: Gaglianone et al. 2010). Grande parte dos trabalhos com as abelhas Centridini lidam com suas relações com as plantas produtoras de óleo. O gênero *Centris* pode visitar todos as famílias de angiospermas produtoras de óleo no Neotrópico, mas *Epicharis* coleta óleo apenas em Malpighiaceae (Machado 2004). *Centris* é conhecido também por visitar Bignoniaceae, Caesalpinaceae, Passifloraceae, Fabaceae, e Sterculiaceae para néctar, e Solanaceae, Caesalpinaceae, Malpighiaceae e Plantaginaceae para pólen (Aguiar 2003; Aguiar et al. 2003). Para coleta de óleo, *Epicharis* visita as malpiguiáceas *Banisteriopsis*, *Bunchosia*, *Byrsonima*, *Heteropterys*, *Mascagnia*, *Peixotoa*, *Stigmaphyllum* e *Tetrapteris*. *Centris* visita estes gêneros também (exceto *Bulchosia*), mas também *Banisteria*, *Dinemandra*, *Dinemagonum*, *Janusia*, *Lophantera*, *Macvaughia*, *Ptilochaeta* e *Trichomaria* (Machado 2004).

O importante papel de Centridini no sucesso reprodutivo de Malpighiaceae Neotropical é inegável, mas não existem casos conhecidos de interações especializadas espécie-espécie (Vogel 1974; Machado 2004). Por outro lado, interações de *Centris* com Plantaginaceae são em alguns casos mais especializadas. *Centris* é conhecido por visitar diversas espécies de Plantaginaceae produtoras de óleo floral, incluindo *Angelonia* (e *Monopera*) (Vogel & Machado 1991; Aguiar & Melo 2009; Martins et al. 2013a), *Monttea* (Simpson et al. 1990) e *Basistemon* (Vogel & Cocucci 1995; Martins et al. 2013a). Espécies do grupo *Centris hyptidis* são proximamente relacionadas a este grupo de plantas (Machado et al. 2002; Martins et al. 2013a). *Centris hyptidis* tem algumas das características mais especializadas entre as abelhas

coletoras de óleo, que são pernas anteriores extremamente longas adaptadas a coleta de óleo nas bolsas da corola de *Angelonia* (Machado et al. 2002). De maneira similar, visitantes de *Monttea aphylla*, que tem elaióforos tricomáticos em sacos (como *Angelonia*), são adaptadas à coleta de óleo com coxins de cerdas ramificadas absorvem o óleo (Simpson et al. 1990). *Centris* também são os principais polinizadores de cerca de 200 espécies de Calceolariaceae (Cosacov et al. 2009), os únicos polinizadores de Krameriaceae (18 species; Simpson et al. 1977), e polinizadores relevantes de *Cypella* (30 espécies em Iridaceae; Vogel 1974) e *Nierembergia* (21 espécies em Solanaceae; Cosacov et al. 2008).

Hipóteses filogenéticas recentes (Straka & Bogusch 2007; Cardinal et al. 2010) e anteriores (Roig-Alsina & Michener 1993) para a família Apidae tem gerado controvérsia sobre a monofilia de Centridini. Usando caracteres larvais, combinados ou não a caracteres de adultos, Roig-Alsina & Michener (1993) e Straka & Bogusch (2007) sugeriram a parafilia de Centridini. Cardinal et al. (2010) também chegaram a essa conclusão trabalhando com dados moleculares. A morfologia de adultos, entretanto, suportam *Centris* e *Epicharis* como grupos-irmãos (Roig-Alsina & Michener 1993), provavelmente devido a caracteres relacionados ao comportamento de coleta de óleo. A filogenia molecular produzida por Cardinal et al. (2010), que incluiu duas espécies de *Epicharis*, seis de *Centris* e 152 outros Apinae, mostra *Epicharis* como irmão de um clado compreendendo *Centris* mais abelhas corbiculadas. As abelhas corbiculadas são aquelas que possuem corbículas, estrutura nas pernas posteriores utilizadas no transporte de pólen (também chamada em inglês, de *polen basket*). É o grupo mais bem conhecido e economicamente importante de abelhas, que consiste em: Euglossini (abelhas das orquídeas), Bombini (*bumblebees*), Meliponini (abelhas sem ferrão), e Apini (abelhas melíferas). Este clado, formado por *Centris*, *Epicharis* e corbiculados, de agora em diante denominado linhagem Apine, também tem sido encontrado em estudos morfológicos (Roig-Alsina & Michener 1993; Silveira 1993; Straka & Bogusch 2007). Porém a morfologia também coloca Anthophorini na linhagem Apine, enquanto os dados moleculares não o fazem. Vale notar que nenhum destes estudos incluíram mais do que oito espécies de *Epicharis* e *Centris*. Um estudo morfológico que incluíu 11 espécies de *Epicharis* e 29 de *Centris*, infelizmente incluiu apenas uma espécie de abelhas corbiculadas e, portanto não foi capaz de testar a monofilia de Centridini (Vivallo 2010).

Cada um destes gêneros, entretanto é consistentemente monofilético em todas as análises acima citadas. Em relação às relações internas de *Centris* e *Epicharis*, três teses de doutorado focaram no grupo (Ayala 1998; Gaglianone 2001; Vivallo 2010), porém nenhum

destes foi publicado até o momento. Uma filogenia bem resolvida do grupo é fortemente necessária para o entendimento da evolução deste grupo e suas interações com as plantas que coletam óleo. Estabelecer uma hipótese filogenética robusta é também essencial para estudos taxonômicos destes conspícuos gêneros, especialmente *Centris* com cerca de 250 espécies.

A grande diversidade deste grupo é uma razão possível para a falta de revisões taxonômicas, mas a ausência de uma filogenia bem resolvida e robusta também tem contribuído para dificuldades taxonômicas. Embora significantes, poucos trabalhos taxonômicos têm sido feitos, considerando a grande diversidade do grupo. Alguns estudos taxonômicos foram publicados nas últimas décadas, as seguintes revisões amplas: *Centris* da América Central e do Norte (Snelling 1974, 1984), subgênero *Wagenknechtia* (Vivallo 2013), o grupo *hyptidis* (Vivallo & Melo 2009), *Paracentris* da América do Sul e *Penthemisia* (Zanella 2002); além de descrições pontuais de novas espécies (Vivallo & Zanella 2012; Vivallo et al. 2013).

O principal objetivo da presente tese foi produzir uma robusta filogenia e datada através de relógio molecular para a linhagem Apine, focando nos grupos viventes de abelhas coletoras de óleo nessa linhagem, *Epicharis* e *Centris*. Assumindo que as extinções não são tão frequentes, uma filogenia datada permitiria inferior se *Centris* e *Epicharis* diversificaram aproximadamente ao mesmo tempo ou se *Epicharis* se diversificou antes de *Centris*. Eu também estava interessada em saber quando e onde (em qual região geográfica provável), o comportamento de coleta de óleo foi perdido e quando as interações com as plantas produtoras de óleo mais jovens, nas famílias Calceolariaceae, Iridaceae, Krameriaceae, Plantaginaceae, e Solanaceae evoluíram. Esses grupos de plantas foram datados através de relógio molecular por Renner & Schaefer (2010), mas eles não conseguiram datar eventos internos de diversificação em *Centris* por que apenas algumas poucas espécies de *Centris* haviam sido sequenciadas até aquele momento.

No capítulo 1, eu apresento a filogenia molecular datada através de calibração fóssil para a família Apidae incluindo 174 espécies e mais de 4000 nucleotídeos alinhados, analisados através de biogeografia e reconstrução de estados ancestrais de caracteres. Os principais resultados que emergem deste estudo são: (i) Centridini, como definido atualmente, é de fato parafilético; (ii) *Centris* é grupo-irmão das abelhas corbiculadas, enquanto *Epicharis* é grupo-irmão de todos, corroborando a linhagem Apine; (ii) esta linhagem se originou possivelmente no Novo Mundo no Cretáceo Inferior, há 91 Ma; (iii) a coleta de óleo se originou no ancestral comum da linhagem Apine e foi perdido no ancestral

das abelhas corbiculadas, além de alguns grupos de *Centris*. O trabalho apresentado no Capítulo 1 está em revisão na revista *Molecular Phylogenetics and Evolution* desde 8 de Abril de 2014.

No capítulo 2 eu apresento os resultados chave das relações internas de *Centris* e *Epicharis*, isto é, entre os subgêneros reconhecidos atualmente e uma discussão acerca das implicações na classificação taxonômica. Eu sugiro rearranjos taxonômicos mais fortes nos subgêneros *Centris* (*Paracentris)* com a revalidação de três subgêneros e a proposição de um novo para *Centris hyptidis* e espécies relacionadas e para o clado sul-americano. Em *Epicharis* todos os subgêneros são monofiléticos, como exceção de *Epicharana*, que inclui *Epicharis s.s.*. Futuras proposições de uma nova classificação baseadas nos presentes resultados necessitariam um estudo mais profundo da morfologia, que facilitaria o reconhecimento dos novos clados propostos. O capítulo 2 será finalizado após a defesa de tese como um artigo em separado para publicação, em revista ainda a ser definida.

A origem da linhagem Apine é congruente com a idade de Malpighiaceae, a família produtora de óleo mais antiga dos Neotrópicos, o que concorda com a origem do aparato coletor de óleo adaptado as flores desta família (padrão *four-legged*), estado ancestral mais provável para este grupo. No capítulo 3, eu analiso o cenário de origens das flores de óleo na Região Neotropical ao longo dos últimos 90 Ma e as trocas de planta hospedeira em *Centris*. Para construir esse quadro evolutivo das flores de óleo e *Centris* eu usei ferramentas de relógio molecular para datar a origem das plantas produtoras de óleo neotropicais, Calceolariaceae, Krameriaceae, Iridaceae, *Nierembergia* (Solanaceae), Plantaginaceae (Capítulo 4) e, para Malpighiaceae, eu usei datação disponível na literatura. Eu encontrei que, enquanto Malpighiaceae se originou no Cretáceo, iniciando o mutualismo com as abelhas coletoras de óleo, as outras plantas começaram a se originar apenas durante o Eoceno, com Krameriaceae e Plantaginaceae. E no Mioceno, outras origens de glândulas de óleo ocorreram em Iridaceae, Solanaceae e Calceolariaceae. Capítulo 3 será finalizado após a defesa de tese como um artigo em separado para publicação, em revista ainda a ser definida.

No capítulo 4 eu apresento a filogenia molecular para as Plantaginaceae neotropicais produtoras de óleo floral, tribo Angelonieae, a segunda fonte de óleo mais importante para *Centris* depois de Malpighiaceae. Angelonieae está entre os grupos com o melhor conjunto de dados sobre a associação com abelhas coletoras de óleo, que aqui eu investigo sob uma perspectiva filogenética para entender a evolução da associação com *Centris*. Eu e meus colaboradores encontramos que Angelonieae se originou nas partes mais secas do sul da

América do Sul no Eoceno médio, simultaneamente com seus principais polinizadores (abelhas do grupo *Trachina*, que incluiu vários subgêneros, Capítulo 2) e trocou para óleo como recompensa a polinizadores quatro ou cinco vezes nos últimos 25 Ma. Os resultados deste capítulo são chave para entender o nicho evolutivo das abelhas coletoras de óleo, que é foco do Capítulo 3. Mas também revelou importantes resultados da sistemática e biogeografia das Plantaginaceae neotropicais. O trabalho apresentado neste capítulo 4 está sob revisão na revista *American Journal of Botany* desde 26 de março de 2014.

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CAPÍTULO 1

THE HONEYBEE/BUMBLEBEE LINEAGE DIVERGED FROM NEW WORLD OIL-COLLECTING BEES: IMPLICATIONS FOR THE ORIGIN OF POLLEN BASKETS

THE CORBICULATE BEES AROSE FROM NEW WORLD OIL-COLLECTING BEES: IMPLICATIONS FOR THE ORIGIN OF POLLEN BASKETS

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ABSTRACT

The economically most important group of bees is the "corbiculates", or pollen basket bees, some 890 species of honeybees (*Apis*), bumblebees (*Bombus*), stingless bees (Meliponini), and orchid bees (Euglossini). Molecular studies have indicated that the corbiculates are closest to the New World genera *Centris*, with 230 species, and *Epicharis*, with 35, albeit without resolving the precise relationships. Instead of concave baskets, these bees have hairy hind legs on which they transport pollen mixed with floral oil, collected with setae on the anterior and middle legs. We sampled two-thirds of all *Epicharis*, a third of all *Centris*, and representatives of the four lineages of corbiculates for four nuclear gene regions, obtaining a well-supported phylogeny that has the corbiculate bees nested inside the *Centris*/*Epicharis* clade. Fossil-calibrated molecular clocks, combined with a biogeographic reconstruction incorporating insights from the fossil record, indicate that the corbiculate clade arose in the New World and diverged from *Centris* 84 (72-95) my ago. The ancestral state preceding corbiculae thus was a hairy hind leg, perhaps adapted for oil transport as in *Epicharis* and *Centris* bees. Its replacement by glabrous, concave baskets represents a key innovation, allowing efficient transport of plant resins and large pollen/nectar loads and freeing the corbiculate clade from dependence on oil-offering flowers. The transformation could have involved a novel function of Ubx, the gene known to change hairy into smooth pollen baskets in *Apis* and *Bombus*.

Key words: ancestral state reconstruction, corbiculate bees, divergence dating, oil-collecting apparatus, molecular phylogeny

INTRODUCTION

Most of the ca. 20,000 species of bees provision their larvae with a mix of pollen and nectar (Danforth et al., 2013; Michener, 2007). Some 450 species in 18 genera and a few families instead provision with a mix of pollen and fatty oils that the females obtain from the flowers of about 2,000 species in 11 families on all continents except Antarctica (Buchmann, 1987; Renner and Schaefer, 2010). By far the largest oil-collecting genus is *Centris* with 230 species, traditionally considered as sister to *Epicharis* (35 species), with both together making up the tribe Centridini (Michener, 2007;

Moure et al., 2012). *Centris* occurs in dry and humid areas of South America, Central America, and southern North America, spanning a latitudinal range from 47° South to 39° North. *Epicharis* occurs only in humid areas from 34° South to 23° North in Mexico. Species in both groups are solitary medium-sized to large bees that build their nests in the soil, either in inclined banks or flat ground or in pre-existing holes in dead trees (Frankie et al., 1993; Gaglianone, 2005). All *Epicharis* and most *Centris* feed their larvae with a mix of pollen and floral oils instead of, or in addition to, nectar (Vinson et al., 2006, 1995). They collect the oil with combs of rigid setae on the anterior and middle pair of legs (Neff and Simpson, 1981; Vogel, 1974). In some *Centris*, the oil-collecting apparatus is restricted to the anterior pair of legs and consists of soft, absorptive hairs. A few have no oil-collecting setae, but all of them have large bristly hind legs (scopae) (Neff and Simpson, 1981; Simpson et al., 1990; Vivallo and Melo, 2009). Based on the morphology of their oil-collecting structures, *Centris* and *Epicharis* are very similar and unique among oil-collecting bees. The only other bees with oil-collecting apparatuses on four legs are the ten species of *Monoeca* (Tapinotaspidini), but their basi-tarsal combs are different from those of *Centris* and *Epicharis* (Neff and Simpson, 1981). Combs on two pairs of legs as present in all *Epicharis* and most *Centris* are associated with the exploitation of New World Malpighiaceae flowers, which have four pairs of oil glands located on the abaxial side of the calyx sepals such that a bee sitting on the flower can exploit them with its four legs while touching the flower's male and female sexual organs (Anderson, 1979; Vogel, 1974). Malpighiaceae are the only oil source for *Epicharis* (Machado, 2004), while species of *Centris* exploit a wider array of oil-offering flowers (Martins et al., 2013; Simpson et al., 1990, 1977; Vogel, 1974).

Adult morphology has traditionally been seen as supporting a sister group relationship between *Centris* and *Epicharis*, i.e., the tribe Centridini (Roig-Alsina and Michener, 1993), but molecular trees have neither consistently nor strongly supported this tribe. Instead, DNA trees that included one or two species of *Epicharis*, up to six of *Centris*, and representatives of other Apidae showed the so-called corbiculates as sister to *Centris* albeit without statistical support (Cardinal et al., 2010; Hedtke et al., 2013; with 58% and 42% maximum likelihood bootstrap values). Cardinal and Danforth (2013), however, recovered *Epicharis* and Centris as sister to the corbiculate bees, supporting the topology of Roig-Alsina and Michener (1993).

The corbiculates, or pollen-basket bees, consist of ca. 890 species, namely the honeybees (Apini, *Apis* with 7-10 species), bumblebees (Bombini, *Bombus* with 240 species), stingless bees (Meliponini, with ca. 450 species), and orchid bees (Euglossini, with 187 species), all with glabrous, concave pollen-carrying hind tibiae. This is the commercially most important clade of bees (Gallai et al., 2009; Klatt et al., 2014). Resolving their sister group and likely time and place

of origin thus is important for interpreting geographic, physiological and genomic evolution of apine bees (Medved et al., 2014). If the corbiculate bees are the sister clade to a monophyletic Centridini, this would imply that oil-collecting setae arose in the common ancestor of the latter, because the next-closest clades do not collect oil. However, if instead the genus *Centris* alone is sister to the corbiculate bees, with *Epicharis* sister to both, this implies that the corbiculates arose from an oil-collecting New World ancestor, with implications for the likely precursor state of the corbicula (Fig. 1)

To answer these questions, we sequenced one ribosomal and three protein-coding nuclear gene regions for 72 of 230 species of *Centris*, 22 of the 35 species of *Epicharis*, and a representative sample of corbiculate bees as well as further outgroups. We then carried out a statistical biogeographic reconstruction on fossil-calibrated versions of the phylogeny of *Epicharis*, *Centris*, and the common ancestor of corbiculates to have a time frame for the gain or loss of oil-collecting setae on three, two, or one pairs of legs.

Fig. 1. Comparative morphology of hindlegs in *Centris*, *Epicharis* and corbiculates. A. Hindleg of female of *Centris* (*Aphemisia*) *plumipes*, showing the scopa. B. Same, inner view of leg. C. Hindleg of *Epicharis (Anephicharis) dejeanii*, outer view. D. Same, inner view. E. Hindleg of an corbiculate bee, *Eufriesea violacea*, showing the tibial corbicula.

MATERIAL AND METHODS

Taxon sampling

We sampled 72 of the 230 species of *Centris* and 22 of the 35 species of *Epicharis* (see Table A1 for species names, collection site, and voucher information), representing all 12 subgenera of *Centris* and all eight of *Epicharis* (Moure et al., 2012), mostly with >2 species, but in the case of

subgenera *Ptilocentris* and *Parepicharis* with only 1 species; subgenera *Anepicharis*, *Cyphepicharis* and *Triepicharis* are monospecific. More than 300 new sequences have been submitted the GenBank (Table A3-a lists all GenBank accession numbers). Other Apinae (Michener's Apidae) representatives are 20 species from the corbiculate tribes Apini, Meliponini, Euglossini, and Bombini and 56 species representing 22 of 33 tribes of Apinae sensu lato (Allodapini, Ammobatini, Ancylini, Anthophorini, Caenoprosopidini, Ceratinini, Ctenoplectrini, Emphorini, Epeolini, Ericrocidini, Eucerini, Exomalopsini, Iseopeolini, Manueliini, Melectini, Nomadini, Osirini, Protepeolini, Rhathymini, Tapinotaspidini, Tetrapediini, Xylocopini). As more distant outgroups, with included eight species from subfamily Megachilinae sensu lato (Michener's Megachilidae) (Fideliini, Pararhophitini, Anthidiini, Lithurgini, Osmiini). In total, our matrix thus comprised 174 species of Apinae and Megachilinae, mostly with sequences from Cardinal et al. (2010). Voucher specimens for our new sequences are housed in the DZUP – Jesus Santiago Moure Entomological Collection at Federal University of Paraná, Brazil, or at the institutions that provided specimens for DNA extractions (Table A1).

Sequence data, alignments, and phylogenetic analyses

Most newly sequenced specimens were field-collected and preserved in EtOH, but some pinned specimens (up to twelve years old) were also used. DNA was extracted using the Qiagen DNeasy blood & tissue extraction kit, following the manufacturer's protocol. We sequenced part of the ribosomal 28S gene (1,400 base pairs) and three nuclear protein-coding genes: LW-Rhodopsin (800 base pairs), Elongation factor 1α – F2 copy (1,000 base pairs), and RNA-polymerase (900 base pairs). Primers sequences and specific conditions are listed in Table A4. Most PCR products were purified and sequenced by Macrogen Inc., South Korea; some were purified with ExoSAP-IT (USB Corporation, Cleveland, OH, USA), for removing leftover primers and dNTPs, using a denaturation temperature of 37°C and an inactivation temperature of 80°C. A mixture of 1.0 µl BigDye® (Applied Biosystems, Foster City, CA, USA), 0.5 µl primer, and 1.5 µl sequencing buffer was then added to this product and, after the sequencing reaction was pipetted onto a sequencing plate of sephadex gel wells, which was run on an ABI 3130 (Applied Biosystems, Foster City, CA, USA). Chromatogram quality evaluation, sequence assembling, and corrections were done in Geneious v. 6.1 (Biomatters, 2013).

All alignments were performed in MAFFT v. 7 (Katoh and Standley, 2013) with default parameters: 200PAM/k=2 scoring matrix for nucleotide sequences; gap opening penalty = 1.53; offset value = 0. The alignment of the protein coding genes EF 1-α and LW-Rhodopsin, both with large introns, relied on the L-INS-i strategy, which is recommended for sequences with multiple

conserved domains and long gaps. The alignment of the RNA polymerase relied on the G-INS-i strategy, recommended for sequences with a global homology. The ribosomal 28S region was aligned based on its secondary structure using the Q-INS-i algorithm in MAFFT v. 7. Minor adjustments were made by eye in Geneious, and we made sure the introns/exon boundaries of EF 1-α and LW-Rhodopsin were maintained. Sequences from the four markers were concatenated in Sequence Matrix v. 1.7.8 (Vaidya et al., 2010).

To assess the homogeneity of base frequencies across taxa, we ran chi-square tests for each of the individual data sets. Results for the four data partitions were: chi-square = 151.04, df = 519, P = 1.0 for the 28S region; 656.58, df = 519, P = 0.000034 for RNA polymerase; 78.56, df = 519, P = 0.05 for rhodopsin; 502.60, df = 519, P = 0.69 for EF1alfa. Because we did four tests on the same data, we applied a more conservative criterion for significance, namely P = 0.01. These results indicate that only the RNA polymerase showed significant heterogeneity in nucleotide composition among taxa. We therefore ran phylogenetic analyses with and without this data partition. We also explored three data partitioning strategies. First, we treated the ribosomal genes as one partition and the protein-coding genes as another; second, we partitioned by gene (28S, RNA-polymerase, LW-Rhodopsin, EF 1-α); third, we partitioned by codon (28S, nt1, nt2, nt3); the introns were added to the third codon partition, as they often exhibit comparable substitution rates to the third nucleotide position. These models were then compared in terms of their likelihood scores. Selection of best-fit models of nucleotide substitutions relied on the Akaike Information Criterion and the Bayesian Information Criterion, as implemented in jModelTest v. 2 (Darriba et al., 2012). Maximum likelihood tree searches and bootstrapping of the combined data using 1000 replicates were performed in RAxML (Stamatakis, 2006) using the graphical interface in raxmlGUI (Silvestro and Michalak, 2012). The concatenated trees were visualized and edited in FigTree v. 1.3 (Rambaut, 2009).

Bayesian tree searches were performed in MrBayes 3.2 (Ronquist et al., 2012) using the CIPRES server (Miller et al., 2010) with four data partitions, one for each gene region (28S, RNA polymerase, LW-Rhodopsin, EF 1-α). The Markov chain Monte Carlo (MCMC) was run for 8 million generations, with trees samples every 1000th generation. We used the default priors for MrBayes, and convergence was assessed with Tracer (Rambaut et al., 2013). Trees obtained prior to convergence of the chain were discarded as burnin (25%), and a 50% majority rule consensus tree was constructed from the remaining 12,000 trees.

Clock models and their calibration

Divergence times were estimated using the Bayesian approach implemented in BEAST 1.7 (Drummond et al., 2012) using a Yule tree prior, the GTR + G substitution model, and the uncorrelated lognormal relaxed clock model, as recommended in the manual when ucld.stdev values in Tracer are ≥0.5. We constrained Anthophorini and Cleptoclade, Xylocopini and the Eucerini, and *Centris*and corbiculates to be monophyletic (Fig. A1; nodes B, C and D) based on the Bayesian and Maximum Likelihood analyses, which consistently showed these clades (Fig. A2 and A3). The MCMC runs, again preformed on the CIPRES server, were 50 million generations long, sampled every 1000th generations. Three separate runs were performed, totaling 250 million generations, and were then combined in LogCombiner (BEAST package). Convergence of the chains was again checked in Tracer. LogCombiner was used to combine all three tree files and to discard 25% of the trees. TreeAnnotator (BEAST package) was used to create a maximum likelihood credibility tree.

Two Apinae fossils were used to calibrate the tree (shown in Fig. A1), using a lognormal prior distribution with the age of the fossils as the offset and a 95% confidence interval as shown in Table A5. We outline our reasoning regarding the use of these instead of other fossils in Table A5. The upper bound for the divergence between Apinae s.l. and Megachilinae s.l. was set to 110 mya, using a mean of 100 and a SD of 5 (Fig. A1, node A), based on the age of this node inferred by Cardinal and Danforth (2013). The latter study enforced a bee maximum age of 145 mya, matching the lower boundary of the Cretaceous. This more or less matches the oldest undisputed angiosperm pollen, which dates to c. 133 mya (Brenner, 1996), although rare angiosperms with cycad-like pollen, as in several early-branching angiosperm families, might easily go unrecognized in the Jurassic or Triassic (Doyle, 1969; Hochuli and Feist-Burkhardt, 2013, 2004; Zavada, 2007).

Ancestral character state reconstruction

In a reduced data set containing only the corbiculates, *Centris*, and *Epicharis*, we inferred the evolution of the oil-collection apparatus using the states: 4-legged apparatus, 2-legged apparatus, and oil combs absent (Table A7). Information on the morphology of oil-collecting apparatuses were gathered from relevant literature (Neff and Simpson, 1981; Snelling, 1984; Vivallo and Melo, 2009; Vivallo, 2013; Vogel, 1974). We reconstructed ancestral states using parsimony and maximum likelihood (Lewis, 2001) in Mesquite v. 2.75 (Madison and Madison, 2011), using the last 1000 trees from a Bayesian chain. We also constrained the important node to its alternative state (e.g., no oil-collecting for the MRCA of *Epicharis* + *Centris* + Corbiculates) and compared the likelihoods of the two reconstructions, using a value of two log units as indicating a significant difference.

Biogeographic analyses

For the biogeographic analysis, the clades were coded as follows: *Epicharis*: Neotropical, based on the occurrence of all 35 species there (Moure et al., 2012); *Centris*: Neotropical and Nearctic, based on the occurrence of the sampled species, which represent the range of the genus

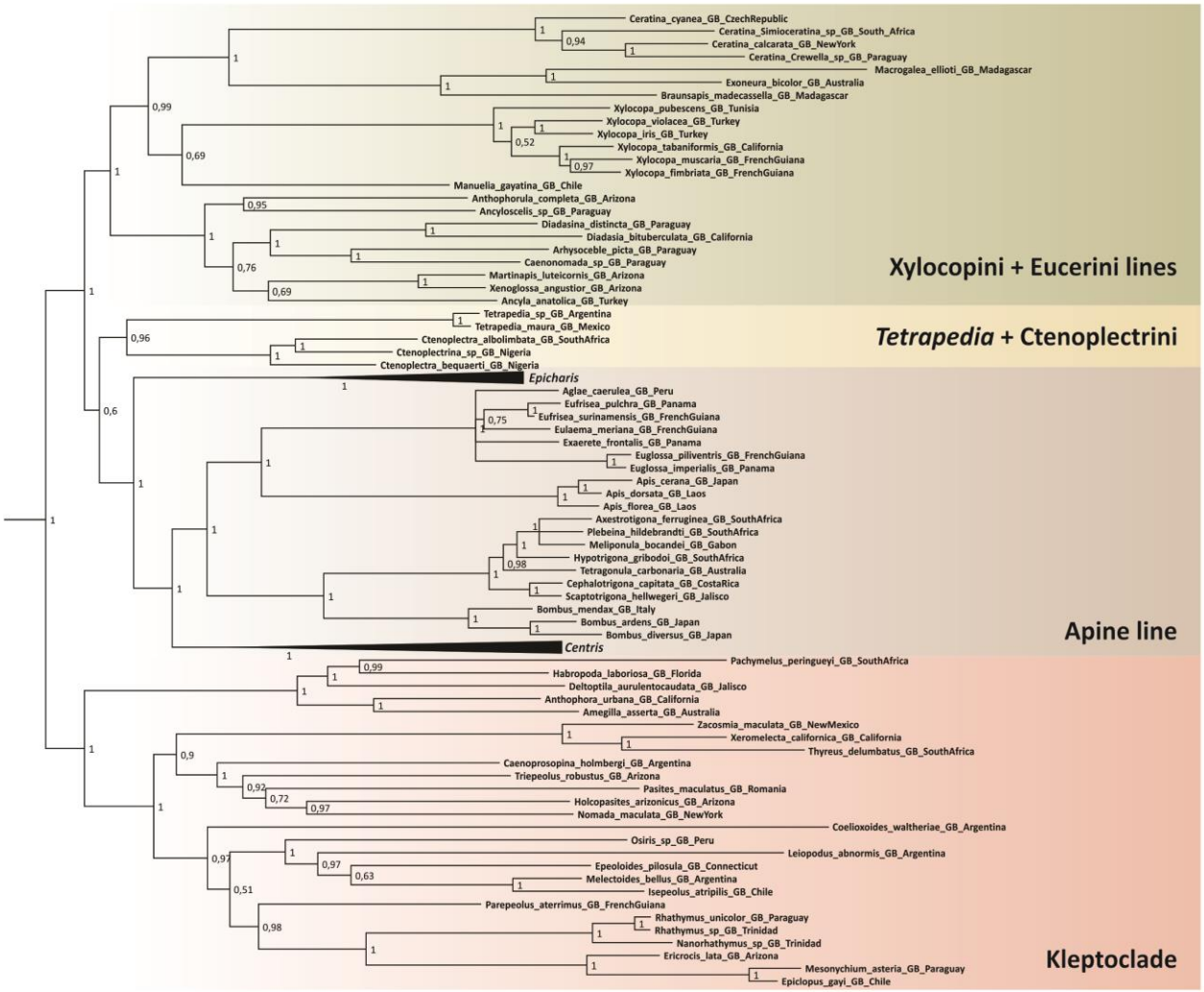

Fig. 2 Bayesian tree with posterior probabilities at nodes resulting from the analysis of 174 taxa and 4300 aligned nucleotides under the gene-partitioning scheme, rooted on Megachilinae as outgroup. The genera *Epicharis* (22 species) and *Centris* (72 species) are collapsed to better shown the other relationships within Apinae. The main groups in Apinae are highlighted.

(Moure et al., 2012); corbiculates, *Bombus*: Palearctic, based on (Hines, 2008, Fig. 2, p.63), which shows the early-branching *Bombus* in the Palearctic; Meliponini: Ambiguous, based on (Rasmussen and Cameron, 2010); these authors leave open whether the stingless bees have a Laurasian origin, given their Nearctic fossils; Euglossini: Neotropics, based on the occurrence of all their species there (Ramírez et al., 2011); *Apis*: Ambiguous because this taxon has fossils from the Nearctic and Palearctic (Engel et al., 2009). We reconstructed ancestral states under parsimony and maximum

likelihood (MK1 model; Lewis, 2001) in Mesquite v. 2.75 (Madison and Madison, 2011), using the fossil calibrated time tree.

RESULTS AND DISCUSSION

Our DNA matrix included the ribosomal 28S gene and three nuclear protein-coding genes (Materials and Methods). Figure 2 shows the Bayesian consensus tree from the combined data, using the gene-partitioning scheme (see also A2 and A3). The two alternative partitioning schemes yielded the same topology for the ingroup, with a slightly higher bootstrap support in the ML reconstruction for the codon-partitioned scheme. Figure 3 shows a fossil-calibrated phylogeny the topology of which is congruent with that of a Bayesian consensus tree and a Maximum Likelihood tree from the same data (Fig. 2 and OSM Fig. A2 and Fig A3). *Centris* is sister to the corbiculate bees, and *Epicharis* sister to both, all with high support. When the RNA polymerase, which was the only data partition with significant heterogeneity in nucleotide composition (see Materials & Methods), was excluded from the ML tree search, we obtained the same topology for the ingroup, but with slightly lower support for the *Centris* + corbiculates clade (73% compared to 89%). Within the corbiculates, *Apis* is sister to Euglossini, and *Bombus* to Meliponini as found in previous studies (Cameron and Mardulyn, 2001; Kawakita et al., 2008; Whitfield et al., 2006). The paraphyly of "Centridini" contradicts results from a morphological cladistics analysis (Roig-Alsina and Michener, 1993) that found several synapomorphies for Centridini, all linked to their oil-collecting apparatuses. A tree in which *Centris* and *Epicharis* are constrained to be monophyletic had a likelihood of -92077, compared to -92068 for the unconstrained tree, a highly significant difference.

Several traditional subgenera within *Centris* and *Epicharis* are not monophyletic, but three main clades in the *Centris* tree are almost congruent with the subgenera *Centris* (*Centris), C*. (*Trachina*) and *C*. (*Melacentris*) (labeled in Fig. A6). The *Centris* and *Trachina* clades each expanded into the Nearctic region during the Miocene, and in North America they now occur mostly in xeric vegetation. They have modified oil-collecting apparatuses adapted to flowers of *Calceolaria*, *Krameria*, and certain Plantaginaceae (Martins et al., 2013; Simpson et al., 1990).

A molecular clock tree calibrated with fossils similar to previous studies (Cardinal and Danforth, 2013; Cardinal et al., 2010; Hines, 2008; Litman et al., 2013; Ramírez et al., 2011; details of the fossils and their placements are provided in online supporting Table A6) indicates that *Epicharis* diverged from the *Centris*/corbiculate clade in the Turonian (early Upper Cretaceous), at 91 (79-102) million years ago (mya), and that the latter diverged from each other at 84 (72-95) mya. The crown age of *Epicharis* is estimated as 28 (18-39) mya, that of *Centris* at 46 (34-58) mya. The

state reconstruction of the oil-collecting apparatus indicates that the ancestral condition in *Centris* is four-legged oil collecting, which was then repeatedly lost (Fig. 3 shows the ancestral condition in *Centris* and the losses of the 4-legged or 2-legged apparatus). In North America, oil-collecting

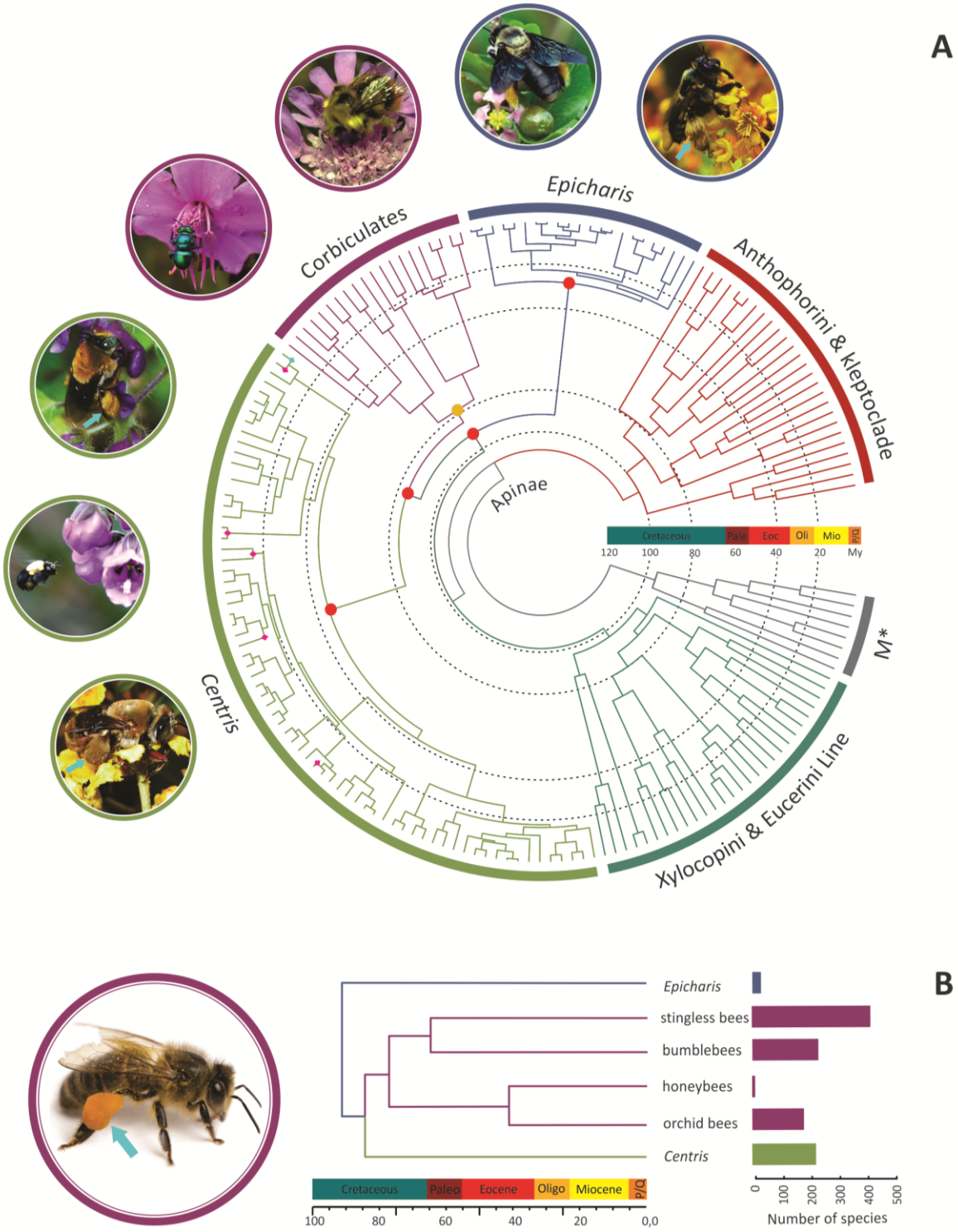

Fig. 3. Time-calibrated phylogeny for corbiculates, *Centris* and *Epicharis*, other Apinae, and Megachilinae, showing bee hind legs, flowers these bees typically forage on, and species diversities. A. Time-calibrated phylogeny (for full species names see Fig. A5). Pie charts indicate the ancestral states of the oil-collecting apparatus inferred over 1000 Bayesian trees, with the colors indicating the most plausible state: four-legged

(purple), absent (yellow). B. Phylogeny for the focal group with the number of species in each of the main clades. Photos counter-clockwise from right: *Epicharis cockerelli* on oil-offering *Byrsonima* (Malpighiaceae), the arrow points the hairy hind leg; *Epicharis flava* on oil-offering *Byrsonima*; *Bombus* on nectar-offering *Knautia arvensis* (Caprifoliaceae); *Apis mellifera* with fully loaded pollen basket; female pollen-collecting *Euglossa* on *Tibouchina* (Melastomataceae); *Centris thelyopsis* on oil-offering *Angelonia pubescens* (Plantaginaceae), the arrow points to the hairy hind leg with an oil-soaked pollen load; *Centris bicolor* on oil-offering *Angelonia eryostachis* (Plantaginaceae), note pollen grains on hairy hind legs; *Centris aenea* on oil-offering *Byrsonima*, the arrow points to a hind leg with a shiny oil load.

behavior was lost at least twice, namely in *Centris* (*Paracentris*) *anomala* at ca. 13 mya, and again in the ancestor of *C.* (*Paracentris*) *pallida* and *C.* (*Paracentris*) *hoffmanseggiae* at ca. 14 mya (Fig. 3). In South America, it was lost in *C.* (*Wagenknechtia*) *muralis* at ca. 5 mya and a second time in *C.* (*Penthemisia*) *tamarugalis* at ca. 2 mya (Fig. 2).

Differently from the North American species, the South American species that lack setae on the hind legs still possess many characters of an oil-collecting ancestor, such as non-functional combs with sparse setae on the forelegs, fitting with their more recent loss of the oil-collecting behavior. Six further species have lost oil-collecting apparatuses in North America (*C. tiburonensis*, *C. vanduzeei*, *C. rhodomelas* and *C. californica*) and South America (*C. mixta* and *C. moldenkei*), but have not been sequenced.

The age of 91 (79-102) mya inferred here for the *Epicharis*/*Centris* clade agrees well with the inferred origin of the Malpighiaceae family at 86 (72-99) mya (Xi et al., 2012), a large plant clade that ancestrally produces oil in four calyx glands (Davis and Anderson, 2010) and that based on a previous molecular clock analysis (with extremely sparse taxon sampling) had been suggested to have co-diversified with *Epicharis* and *Centris* (Renner and Schaefer, 2010). The nesting of the corbiculate lineage inside an oil-collecting clade implies that the ancestral provisioning behavior in corbiculates involved the collecting and transporting of oil in setae on all pairs of legs (Fig. 1 and 3). The transition from setose hind leg tibiae to glabrous corbiculae may relate to the use of resins in nest construction as true of many tropical corbiculate bees (Michener, 2007; Simone-Finstrom and Spivak, 2010). Except for bumble bees, females of all corbiculates harvest plant resins, carrying them back to the nests in their concave pollen baskets (Noll, 2002; Simone-Finstrom and Spivak, 2010). While suitable for carrying oils, bristle-bearing tibiae as found in all *Centris* would not work for transporting sticky resin pellets back to the nest. The main gene determining the formation of a smooth, concave, bristle-free hind tibia in honeybees (*Apis mellifera*) and bumblebees (*Bombus impatiens*) is Ultrabithorax (*Ubx*); interruption of *Ubx* by RNAi transforms a bristle-free basket into

one covered with bristles (Medved et al., 2014). This suggests that the evolution of a glabrous pollen carrying apparatus may be due to the acquisition of a novel role of *Ubx* in the suppression of bristles on the hind leg tibia (Medved et al., 2014). The females of both *Centris* and *Epicharis* have unusually broad and flat basitarsi in their hind legs (Fig. 1). The genetic machinery to produce such "flat legs" apparently was already in place in the ancestral lineage of the corbiculates and probably facilitated the transition to their flat and glabrous hind tibiae.

The *Epicharis* lineage, which relies entirely on the flowers of Malpighiaceae and therefore always has oil-collecting setae on four legs, today comprises just 35 species in the humid tropics; it extends no further north than central Mexico (Moure et al., 2012). By contrast, some *Centris* species no longer depend on Malpighiaceae, thereby becoming free to reduce the setae on their middle legs. Today, this genus has 230 species and ranges into North America and the Caribbean, occupying from humid to xeric habitats. The early-diverging *Centris*, however, all have oil-collecting setae on four legs and co-evolved with the four-gland-flowers of Malphigiaceae over the past 90 mya.

Biogeographically, the nesting of corbiculate bees inside a New World clade implies that the most recent common ancestor of the corbiculates/*Centris* lineage probably lived in the Americas (Fig. A5), as also inferred by Hedtke et al. (2013) albeit without statistical support for the relevant nodes. Such an origin has never so far been suspected based on the fossil record of corbiculates, which stems mostly from the Northern Hemisphere in Europe and North America, and from Caribbean amber and dates back to the Latest Cretaceous, ca. 72 mya (Engel, 2000), fitting with our inferred corbiculate stem age of 84 (72-95) mya. Cardinal and Danforth (2011) found that the complex social behaviors that evolved twice in the corbiculate bees (once in the honeybee, once in the stingless bees) have evolved over an 80 million year timespan. The present study now adds the insight that the evolution of the pollen basket, the key trait of corbiculates allowing them to carry pollen mixed with nectar, apparently preceded the evolution of eusociality by just a few million years.

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SUPPLEMENTARY INFORMATION

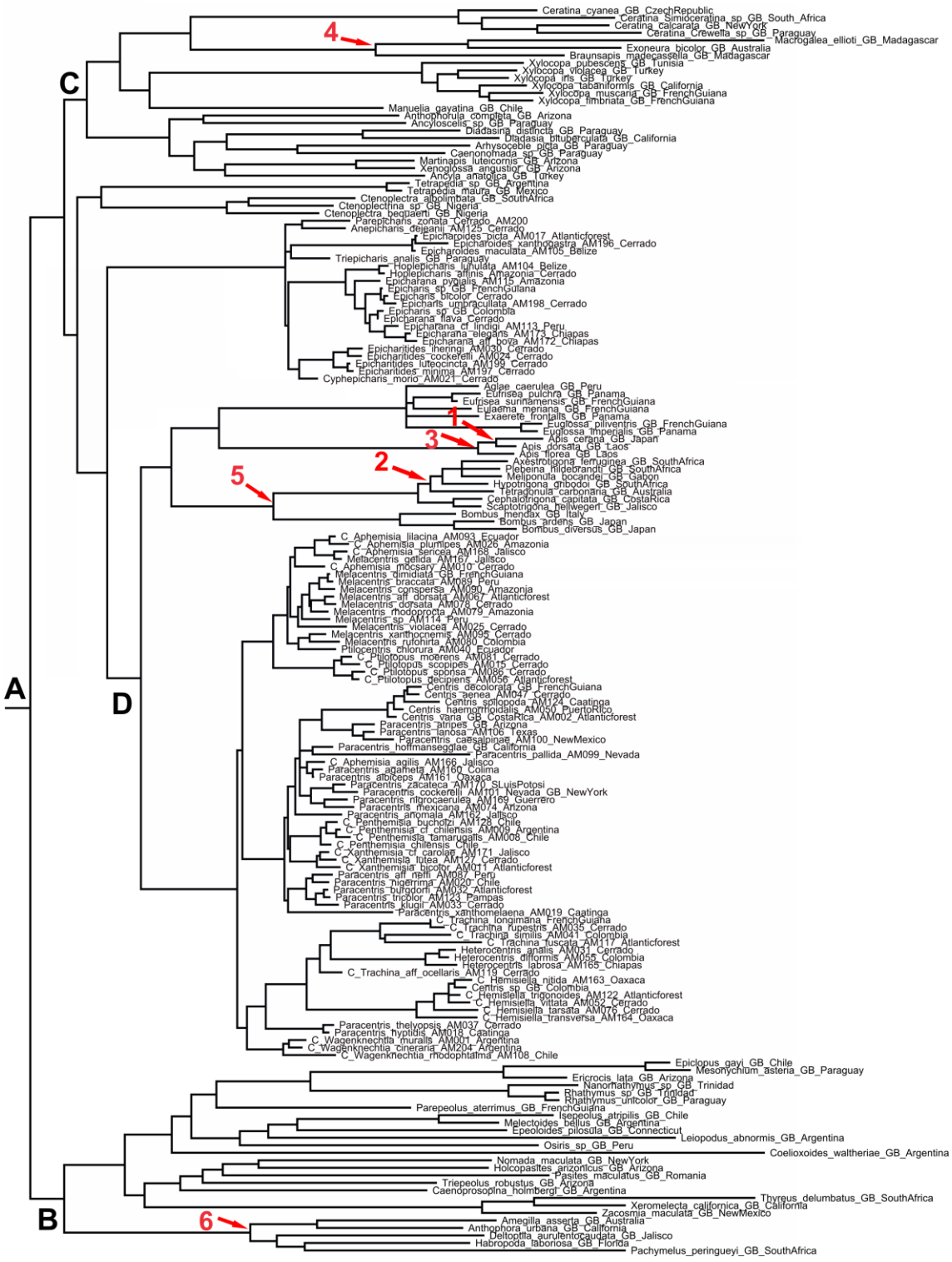

Fig. A1: Bayesian consensus tree showing the placement of the fossil calibration points (1, 2) as well as the fossils that provided cross validation (3, 4, 5, 6) as explained in Table S5; 1 = *Apis lithohermaea* Engel, 2006; 2 = *Kelneriapis eocenica* (Kelner-Pillault, 1970); 3 = *Apis henshawi* Cockerell, 1907; 4 = *Boreallodape* sp; 5 = *Cretotrigona prisca* (Michener & Grimaldi, 1998); 5. *Paleohabropoda oudardi* Michez & Rasmont, 2009. Full literature cited in Table S5. Clades A-D were recovered here and in the maximum likelihood tree, but were constrained to be monophyletic in the dating runs.

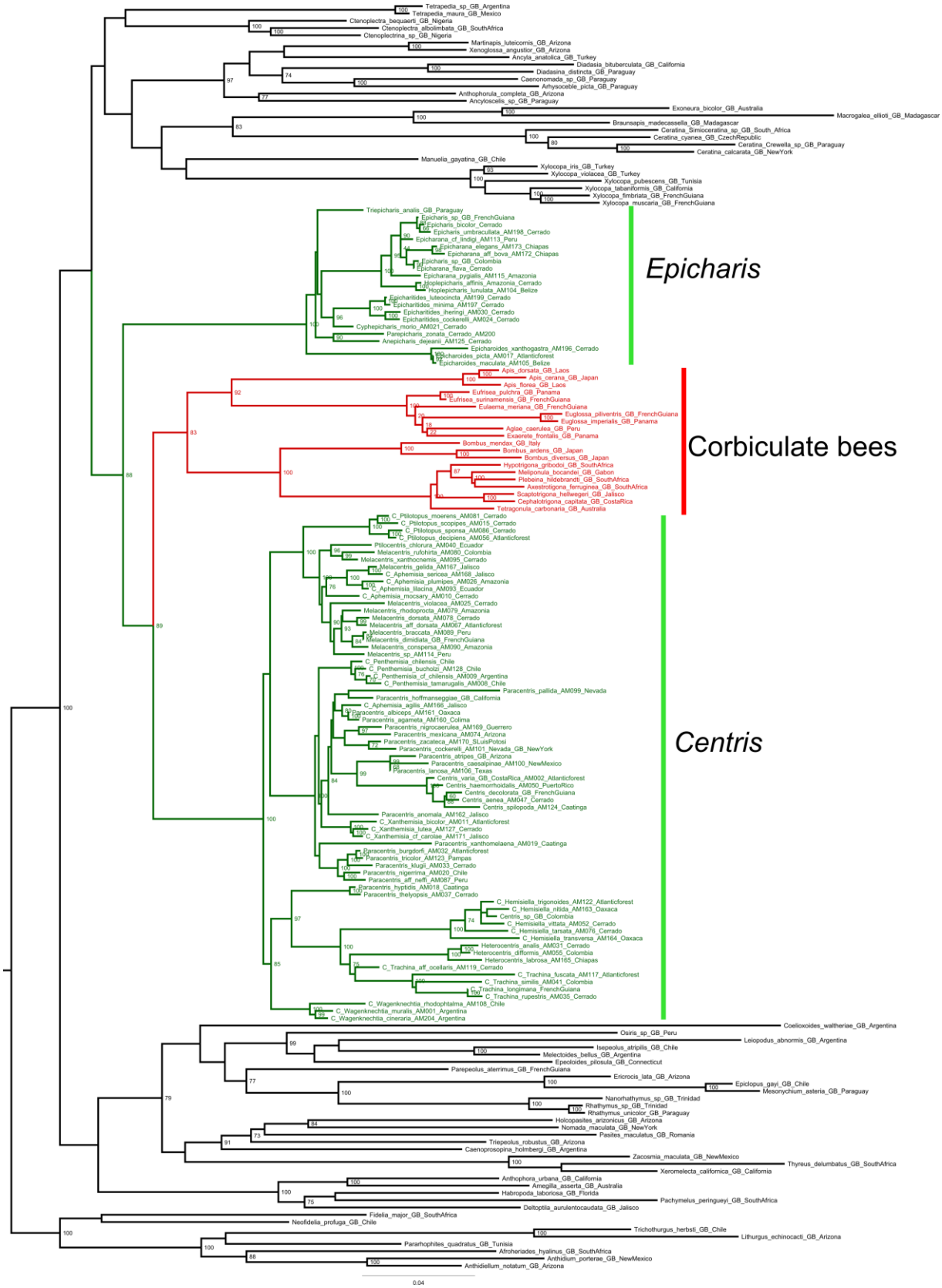

Fig. A2: Maximum likelihood tree resulting from the analysis of the complete data (174 taxa and 4300 aligned nucleotides), rooted between Apinae and Megachilinae. The genera *Epicharis* and *Centris*, and the corbiculate bees are highlighted. Maximum likelihood bootstrap support values ≥70 are shown at nodes.

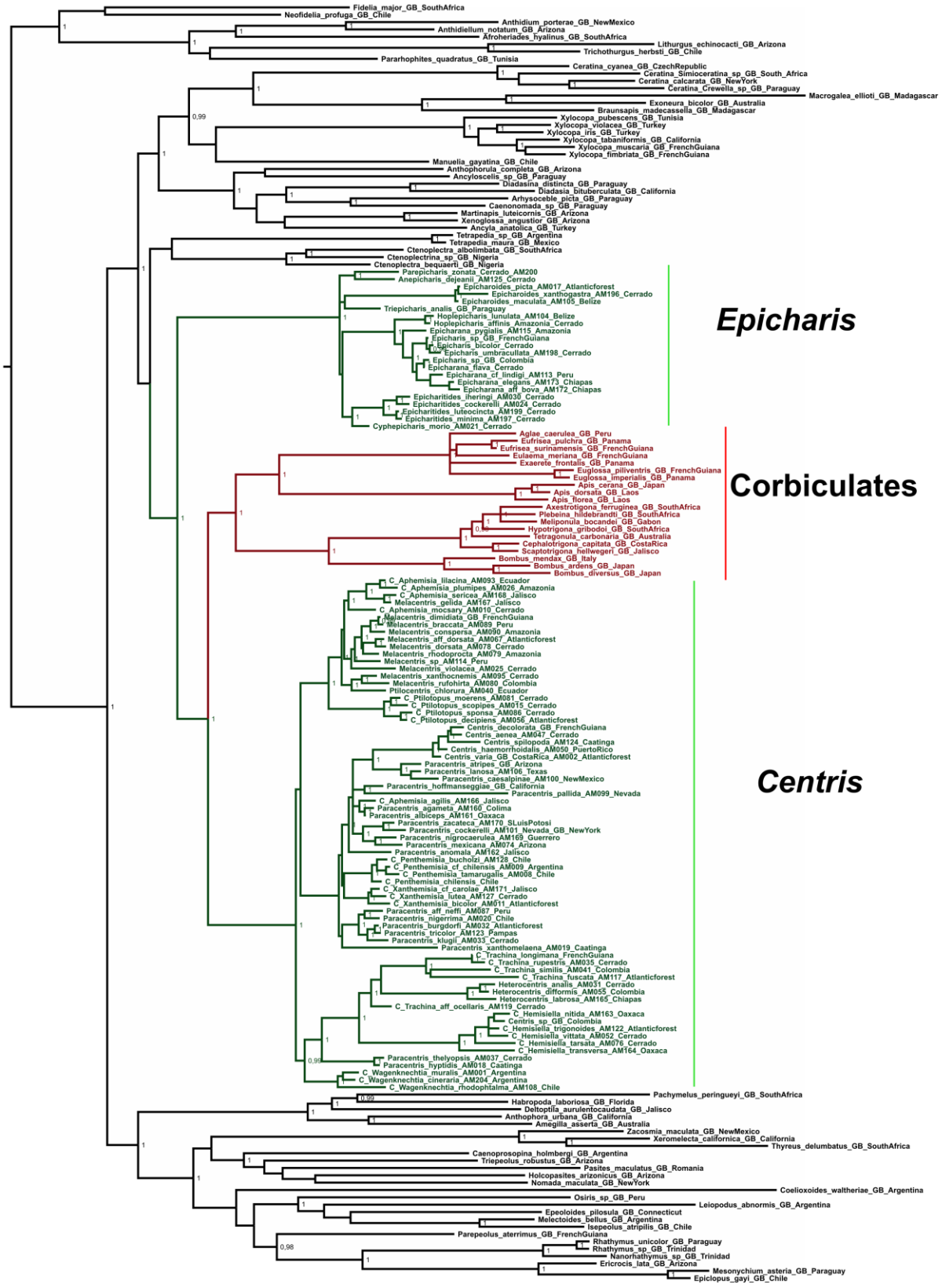

Fig. A3: Bayesian consensus tree resulting from the analysis of the complete data (174 taxa and 4300 aligned nucleotides), rooted between Apinae and Megachilinae. The genera *Epicharis* and *Centris*, and the corbiculate bees are highlighted. Posterior probability values ≥98 are shown at nodes.

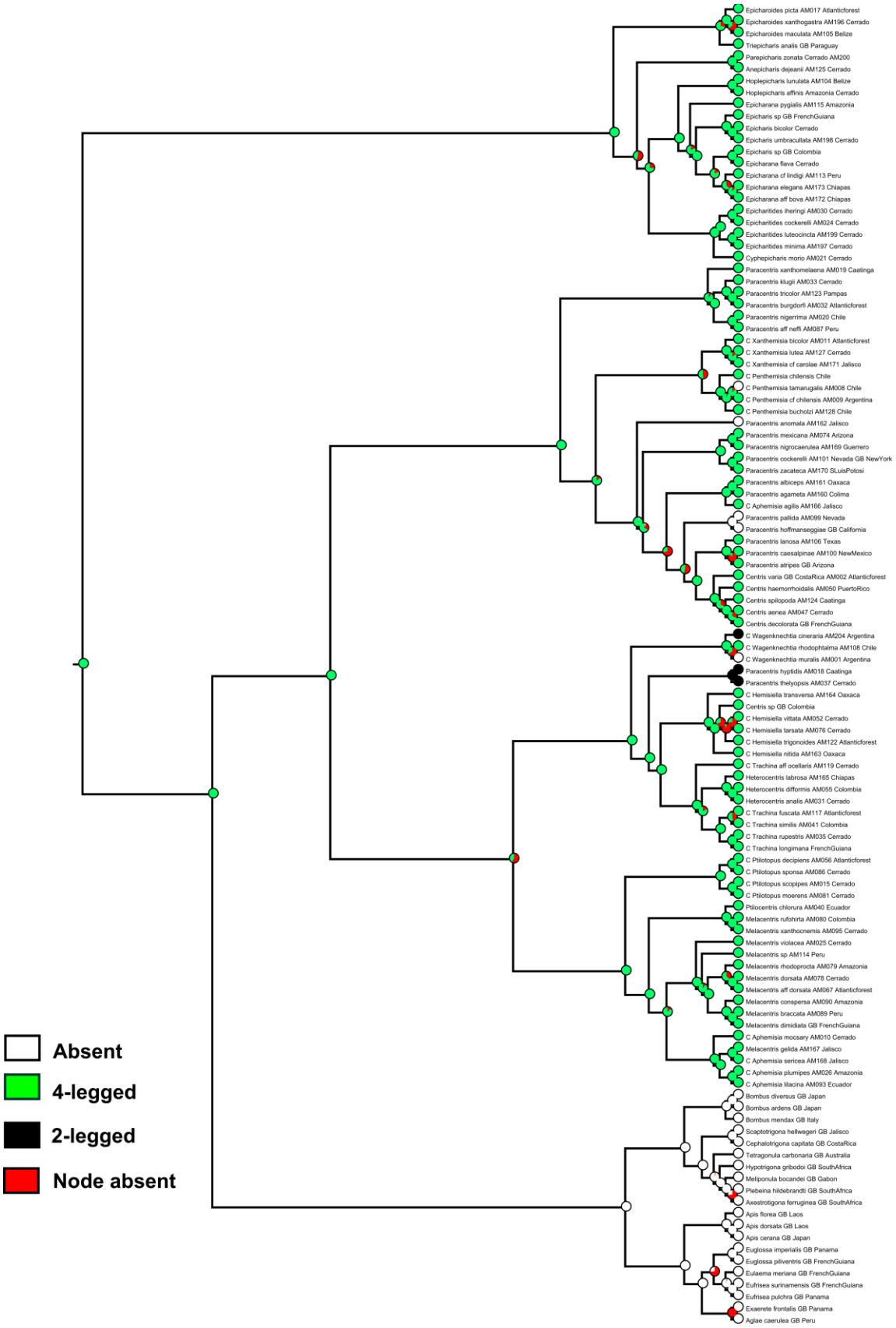

Fig. A4. Ancestral state reconstruction of the morphology of the oil-collecting apparatus in *Epicharis*, *Centris*, and the corbiculate bees on a randomly selected Bayesian tree (from the stationary phase), using the states: absent (white); four-legged (green); and two-legged (black). Pies at nodes represent the portion of 1000 Bayesian

trees that showed the respective state. Wedges colored in red indicate the proportion of the 1000 trees in which the respective node was absent.

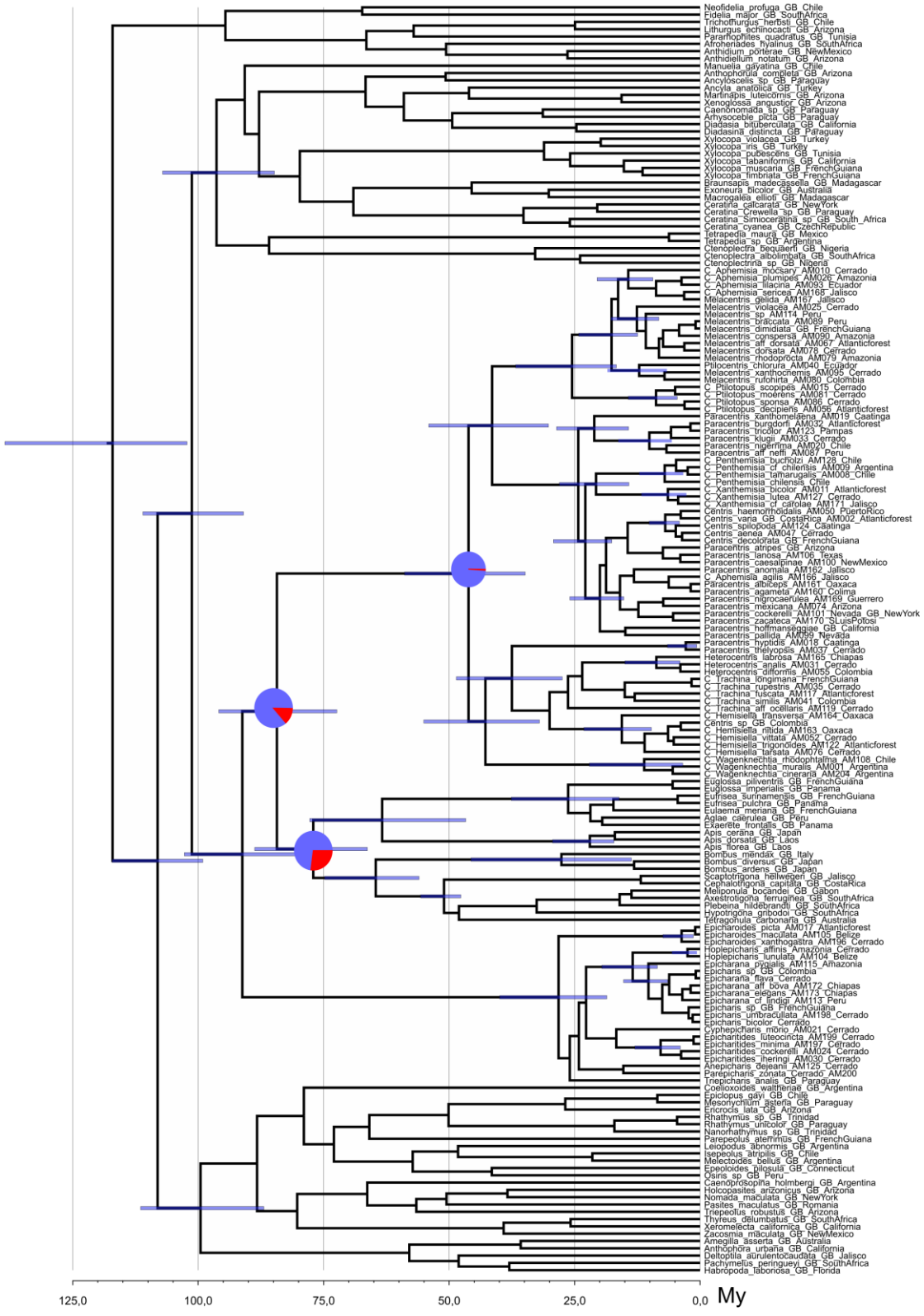

Fig. A5. Bayesian maximum clade credibility time tree for 72 *Centris*, 22 *Epicharis*, 20 corbiculates, and 54 other representatives of Apinae and 8 Megachilinae as outgroups, obtained under a relaxed clock model. For all significantly supported nodes, bars show the 95% Highest Posterior Density intervals around the estimated ages. The pie diagrams refer to the reconstructed biogeographic occurrence under maximum likelihood, with purple referring to an ancestral distribution in the Neotropics and red to the other three states, Palearctic, Nearctic, or ambiguous (see *Materials and Methods*).

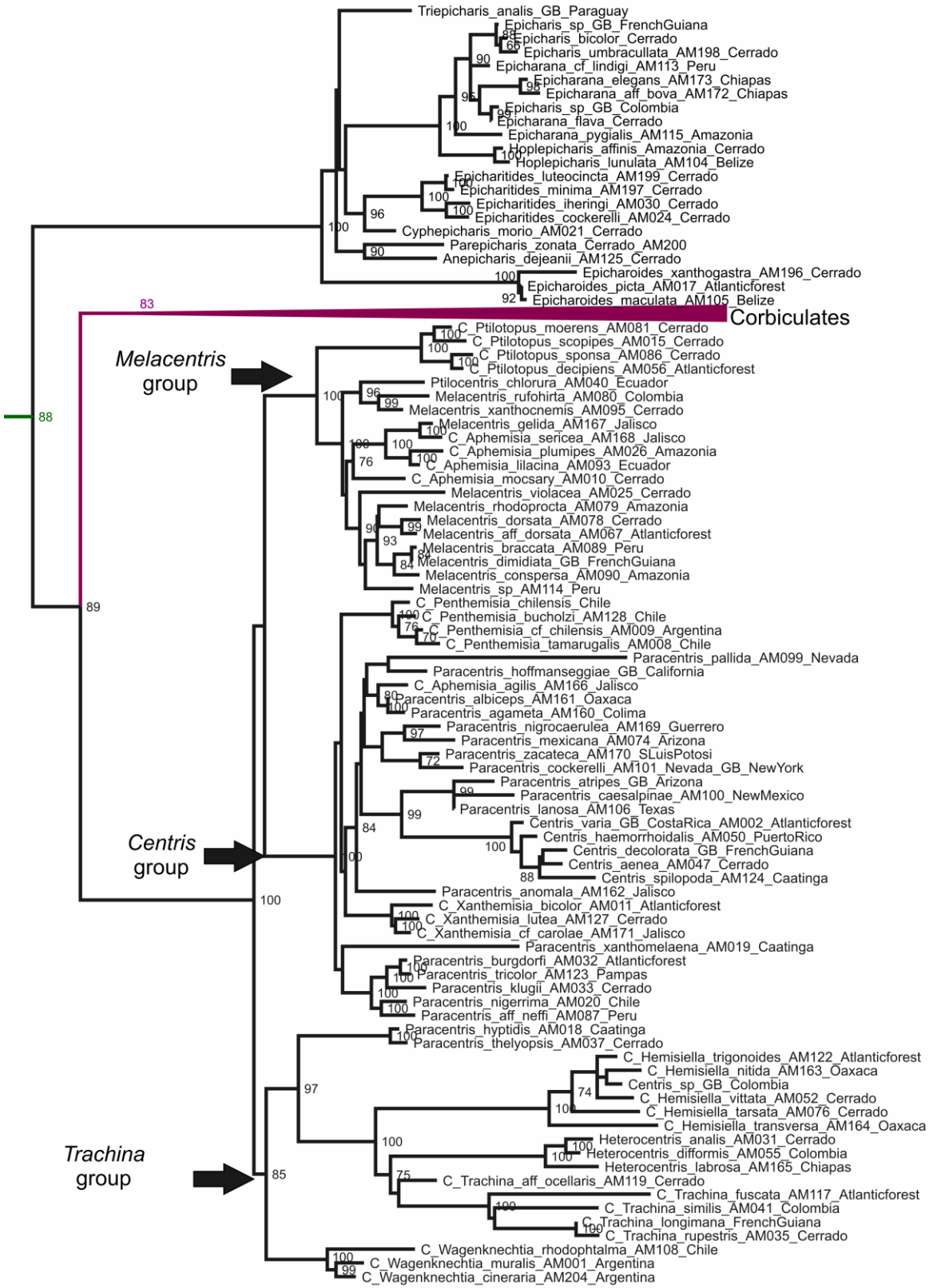

Fig. A6. Detail of the maximum likelihood tree resulting from the analysis of the complete data (Fig. S2), showing taxonomic subgroups in *Centris:* group *Melacentris*, group *Centris* and group *Trachina*.

Table A1. List of species used in this study with author names and collection data

a) Species in *Centris* and *Epicharis*, newly sequenced for this study, ordered by traditional subgeneric classification (see Moure et al. 2012)

Species	**Collecting data**	**Collector**	**DNA Code**	**Entomological Collection* and Reference number**
Centris (Aphemisia) lilacina Cockerell, 1919	Ecuador, Napo, Tiputini Biol. Station, 1-15 Jul. 2000	Cameron	AM093	CR
Centris (Aphemisia) mocsaryi Friese, 1899	Brazil, Minas Gerais, Sacramento, 22 Mar. 2008	L.C. Rocha-Filho	AM010	DZUP 27442
Centris (Aphemisia) plumipes Smith, 1874	BR, Amazonas, Barcelos, Rio Padauari, com. Acuquaia, 8 – 10 Jun. 2010, 1°13’36’’N, 63°59’20’’W	O. Mielke & M. Casagrande	AM026	DZUP 27457
Centris (*Aphemisia*) *agilis* Smith, 1874	ECO-TAP-E95722/Mex., Jal., Tamazula 1857 m/19.83688 N, 103.30157 W/31/10/2012, 11:30	J. Mérida	AM166	DZUP25639
Centris (*Aphemisia*) *sericea* Friese, 1899	Mexico, Jalisco, 20km SW de Tolimán, 02 Aug. 2013, 19.4336°N, 103.9843°W, 1046 m	G. Melo & B. Rosa	AM168	DZUP 25645
Centris (Centris) varia (Erichson, 1849)	Brazil, São Paulo, Picinguaba, 10 Apr. 2008	L.C. Rocha-Filho	AM002	DZUP 27435
Centris (Centris) aenea Lepeletier, 1841	Brazil, GO, Alto Paraíso de Goiás, 1 May 2010, 14º 19’S, 47º30’W, 1089 m	A. Aguiar	AM047	UNB 5104
Centris (Centris) haemorrhoidalis (Fabricius, 1775)	Puerto Rico, Mar. 2004	S.A. Cameron	AM050	CR
Centris (Centris) spilopoda Moure, 1969	Brazil, Bahia, 3 km N de Itacarés, 10 m, 14º94'S, 39º01'W, 9 Jan. 2007	G. Melo	AM124	DZUP 27502
Centris (Hemisiella) vittata Lepeletier, 1841	Brazil, GO, Teresina de Goiás, 13º34’39.3’’S, 47º11’07.8’’W, 422 m 02 Apr. 2010	A. Aguiar & A. Martins	AM052	DZUP 27471
Centris (Hemisiella) tarsata Smith, 1874	Brazil, GO, Teresina de Goiás, 13º34’39.3’’S, 47º11’07.8’’W, 422 m 02 Apr. 2010	A. Aguiar & A. Martins	AM076	UNB 5108

Centris (Hemisiella) trigonoides Lepeletier, 1841	Brazil, Rio Grande do Sul, Porto Alegre, Morro do Osso, 30 Oct. 2012	A. Aguiar	AM122	UNB 24030
Centris (Hemisiella) nitida Smith, 1874	Mexico, Oaxaca, 8km W de San Juan Bautista Cuicatlan, 24 Aug. 2013, 17.8008°N, 97.0341°W, 1536 m.	G. Melo & B. Rosa	AM163	DZUP 25650
Centris (Hemisiella) transversa Pérez, 1905	México, Oaxaca, 8km W de San Juan Bautista Cuicatlan, 24 Aug. 2013, 17.8008°N, 97.0341°W, 1536 m.	G. Melo & B. Rosa	AM164	DZUP 25651
Centris (Heterocentris) analis (Fabricius, 1804)	Brazil, Goiás, Mundo Novo, Margem do rio Crichás,13°54'55"S 49°56'58"W, 258 m 28.iv.2010	A. Aguiar	AM031	DZUP 27459
Centris (Heterocentris) difformis Smith, 1854	Colombia, Amazonas, Leticia, km 14, via Tarapaca, 04º07'105"S 69º57'298"W, 200 m, 4 Sep. 2001	M. Sharkey & D. Campos	AM055	CR
Centris (Heterocentris) labrosa Friese, 1899	México, Chiapas, 9 km NE de Chiapa de Corzo, 14 Aug. 2013, 16.7237°N, 92.9247°W, 898 m,	G. Melo & B. Rosa	AM165	DZUP 25642
Centris (Melacentris) violacea Lepeletier, 1841	Brazil, Goiás, Campo Alegre, BR-050, 27 Jul. 2010	A. Aguiar	AM025	UNB 5100
Centris (Melacentris) dorsata Lepeletier, 1841	Brazil, GO, Cavalcante, 13º46’36’’S, 47º24’42’’W, 802 m, 03 Apr. 2010	A. Aguiar & A. Martins	AM078	DZUP 27487
Centris (Melacentris) rhodoprocta Moure & Seabra, 1960	Brazil, Acre, Mâncio Lima, Pq. Nac. Serra do Divisor, Porção Norte, 200-400 m, 7º26'S 73º39'W, 10-21 Sep. 2011	D. Dolibaina & D. Moura	AM079	DZUP 167753
Centris (Melacentris) rufohirta Friese, 1900	Colombia, Amazonas, Leticia, km14 via Tarapaca, 04.07.105'S 69 57.208'W, 200 m, 4 Sep. 2001	M. Sharkey & D. Campos	AM080	CR
Centris (Melacentris) braccata Packard, 1869	Peru, SM, Moyombamba, S0601/W7659, 930 m, 18 Apr. 03	C. Rasmussen	AM089	CR

Centris (Melacentris) conspersa Mocsáry, 1899	Brazil, Amazonas, Novo Airão, Pousada Bela Vista, 38 m, 2º37'33"S 60º56'36" 4 Jul. 2008	M. G. Hermes	AM090	DZUP 27489
Centris (Melacentris) xanthocnemis (Perty, 1833)	Brazil, Goiás, Alto Paraíso de Goiás, 11 Feb. 2012, 14º15'05"S, 47º29'40"W	A. Aguiar	AM095	UNB 5083
Centris (Melacentris) aff. *dorsata* Lepeletier, 1841	Brazil, Paraná, Morretes, 15 Feb. 2006	F. Vivallo	AM067	DZUP 27481
Centris (*Melacentris.*) sp.	Peru, Cusco, Quincemil, 1 Sep. 2012	B. Rosa	AM114	DZUP 27496
Centris (*Melacentris*) *gelida* Snelling, 1984	ECO-TAP-E96636/Mex., Jal., Talpa/Cumbre de Guadalupe, 2119 m/29.16791 N, 104.71288 W/06/11/2012, 12:20 hrs	Jorge Mérida	AM167	DZUP25638
Centris (Paracentris) agameta Snelling, 1974	Mexico, Colima, 12km E de Minatitlán, 02 Aug. 2013, 19.3565°N, 103.9376°W, 676 m	G. Melo & B. Rosa	AM160	DZUP 25643
Centris (Paracentris) albiceps Friese, 1899	México, Oaxaca, 8km W de San Juan Bautista Cuicatlan, 24 Aug. 2013, 17.8008°N, 97.0341°W, 1536 m	G. Melo & B. Rosa	AM161	DZUP 25649
Centris (Paracentris) anomala Snelling, 1966	ECO-TAP-E-95608/Mex. Jal. Valle de Juárez/Cerro Alto, 2258 m/19.91541 N, 102.95975 W/20/10/2012, 13:40 hrs	Jorge Mérida	AM162	ECO-TAP E95608
Centris (Paracentris) nigrocaerulea Smith, 1874	ECO-TAP-E-97770/Mex., Gro., Chilapa/Chilapa, 1999 m/17.7011N, 9912556W/28/11/2012, 12:00 hrs	Oscar Martínez López	AM169	DZUP25641
Centris (Paracentris) zacateca Snelling, 1966	México, San Luis Potosí, 16km SW de San Luis Potosi, 09 Aug. 2013, 22.0898°N, 101.1302°W, 2184 m	G. Melo & B. Rosa	AM170	DZUP 25644
Centris (Paracentris) cockerelli Fox, 1899	USA, NV, Clark Co. 0.6 mi/ E. Calville Point 1249 ft/11S E708846 N400125/7 Apr. 2004, on *Krameria erecta*	L. Saul	AM101	BBSL 539628
Centris (Paracentris) hyptidis Ducke, 1908	Brazil, GO, Teresina de Goiás, 13°34'39,3"S, 7°11'07,8"W,422 m, 02 Apr. 2010	A. Aguiar & A. Martins	AM018	DZUP 27450

Centris (Paracentris) xanthomelaena Moure & Castro, 2001	Brazil, Bahia, Ipirá, Santa Quitéria, 6 Jan. 2010	K. Ramos & V. Kanamura	AM019	DZUP 27451
Centris (Paracentris) nigerrima (Spinola, 1855)	Chile, IV Região, Pisco Equi, 30 Sep. 2008, S30°07'23'', W70°29'34'', 1265 m	K. Ramos	AM020	DZUP 27452
Centris (Paracentris) burgdorfi Friese, 1900	Brazil, Paraná, Balsa Nova, Rio dos Papagaios, 23 Feb. 2008, 25º28' 49º46'W	M.G. Hermes	AM032	DZUP 27460
Centris (Paracentris) klugii Friese, 1899	Brazil, MG, São Gonçalo do Rio Preto, 1353 m, 18º11'S, 43º00'W, Sep. 2008	A. Aguiar & A. Martins	AM033	DZUP 27461
Centris (Paracentris) thelyopsis Vivallo & Melo, 2009	Brazil, GO, Teresina de Goiás, 405 m, 13º34'48''S, 47º11'12''W, 01 Apr. 2010	A. Aguiar & A. Martins	AM037	DZUP 27465
Centris (Paracentris) mexicana Smith, 1854	USA, Arizona: Cochise / Country: Mule Mountains, Dixie Canyon / 31°28.6'N 109°50'W / 20 Jul. 2009	V. Ahrens & W. Pulawski	AM074	CAS
Centris (Paracentris) pallida Fox, 1899	USA, NV, Clark Co. 1 mi/ NW Stump Spr./ 2867 m, 115 E604827/ N3983893 9 Jun. 2004/ Net, on *Stanleya pinnata*	S. Higbee	AM099	BBSL 531233
Centris (Paracentris) caesalpinae Cockerell, 1897	Socorro Co.NM/Sevilleta NWR/5 pts Creosote/17 May 2001/Sweep Net.	K. Wetherill	AM100	BBSL 708632
Centris (Paracentris) lanosa Cresson, 1872	Texas: Bastrop Co./Stengl-Lost Pines Res. Sta./30.080 N 97.183 W/12 May 2006	J.L. Neff	AM106	J.L. Neff 31132
Centris (Paracentris) tricolor Friese, 1899	Brazil, Rio Grande do Sul, Caçapava do Sul, Guaritas, 31 Oct. 2012	A. Aguiar	AM123	UNB 24031
Centris (Paracentris) aff. *neffi* Moure, 2000	Perú, JU, Concepción, Huaycha, btw Orcotuna and Mito, 3280 m, S 11º56'897", W 75º19'381", 22.i.03	C. Rasmussen	AM087	CR
Centris (Penthemisia) chilensis (Spinola, 1851)	Chile, V Region, Valparaiso, 18 Sep. 2002	F. Vivallo	AM007	DZUP 27439
Centris (Penthemisa) chilensis (Spinola, 1851)	Chile, IV Región, Tongoy, Playa de Socos, 1-2 Oct. 2008, 30º15'14"S 71º29'21" W	K. Ramos	AM066	DZUP 27480

Centris (Penthemisia) cf. *chilensis* (Spinola, 1851)	Argentina, Mendoza, Mendoza, 10 Oct. 2005	F. Vivallo	AM009	DZUP 27441
Centris (Penthemisia) tamarugalis Toro & Chiappa, 1989	Chile, Tamarugal, viii.2007	F. Vivallo	AM008	DZUP 27440
Centris (Penthemisia) buchholzi Herbst, 1918	Chile: Coquimbo (Region IV)/km marker 562.5 on Rt. 5 (north of La Higuera) 20 Oct. 2009	J. Litman	AM128	UNB 24032
Centris (Ptilocentris) chlorura Cockerell, 1919	Ecuador, LO, Loja, Jardin Botanico Reinaldo Espinosa, 19 Dec. 02, 04°02S/79°12 W, 2150 m	C. Rasmussen	AM040	CR
Centris (Ptilotopus) scopipes Friese, 1899	Brazil, GO, Cavalcante, 13°46’36’’S, 47°22’42’’W 802 m, 03 Apr. 2010	A. Aguiar & A. Martins	AM015	DZUP 27447
Centris (Ptilotopus) decipiens Moure & Seabra, 1960	Brazil, Paraná, Morretes, 15 Mar. 2006	F. Vivallo	AM056	DZUP 27473
Centris (Ptilotopus) moerens (Perty, 1833)	Brazil, GO, Teresina de Goiás, 471 m, 13º15’47’’S, 46º56’39.1’’W	A. Aguiar & A. Martins	AM081	UNB 5109
Centris (Ptilotopus) sponsa Smith, 1854	Brazil, GO, Teresina de Goiás, 405 m, 13º34’48’’S, 47º11’12’’W, 01 Apr. 2010	A. Aguiar & A. Martins	AM086	UNB 5110
Centris (Trachina) rupestris Azevedo & Silveira, 2005	Brazil, MG, São Gonçalo do Rio Preto, Sep. 2008	A. Aguiar & A. Martins	AM035	DZUP 27463
Centris (Trachina) similis (Fabricius, 1804)	Colombia,Amazonas, Leticia, km14 via Tarapaca, 04.07.105'S 69 57.208'W, Red. 200m, 4 Sep. 2001	M. Sharkey & D. Campos	AM041	CR
Centris (Trachina) fuscata Lepeletier, 1841	Brazil, Rio de Janeiro, Santa Maria Madalena, 10 Sep. 2011	G. Melo	AM117	DZUP 27499
Centris (Trachina) aff. *ocellaris* (Ducke, 1906)	Brazil, Goiás, Alto Paraíso de Goiás, Vila de São Jorge, 22 Sep. 2012	A. Aguiar & A. Martins	AM119	UnB 24033
Centris (Wagenknechtia) muralis Burmeister, 1876	Argentina, Mendoza, Mendoza, 12 Oct. 2005	F. Vivallo	AM001	DZUP 27434

Centris (Wagenknechtia) rhodophthalma Pérez, 1911	Chile: Coquimbo/Limari: Combarbala,/14 km N, 905 m/31.145 S 71.105 W/21 Oct. 2011	J.L. Neff	AM108	JL Neff 35137
Centris (Wagenknechtia) cineraria Smith, 1854	Estancia Verdadera Argentina - Provincia Santa Cruz, -50.842694 -72.224805, 329 m, 11 Dec. 2013	A. Sérsic	AM204	AAC
Centris (Xanthemisia) bicolor Lepeletier, 1841	Brazil, Paraná, Curitiba, 9. Mar. 2008	F. Vivallo	AM011	DZUP 27443
Centris (Xanthemisia) cf. *carolae* Snelling, 1866	ECO-TAP-E-96638/Mex. Jal.,Talpa/Cumbre de Guadalupe, 2119 m/20.16791 N, 104.71288W/06/11/2012, 12:20./ Col. Jorge Mérida	J. Mérida	AM171	ECO-TAP E96638
Centris (Xanthemisia) lutea Friese, 1899	Brazil, Bahia, 24 km a SW de Encruzilhada, 15.710ºS 41.042ºW, 900 m, 20 Dec. 2012	G. Melo & P. Grossi	AM127	DZUP 25627
Epicharis (Anepicharis) dejeanii Lepeletier, 1841	Brazil, Distrito Federal, Riacho Fundo, Oct. 2012	D. Oliveira	AM125	UNB 24034
Epicharis (Cyphepicharis) morio Friese, 1924	Brazil, Minas Gerais, Virginia, Fazenda dos Campos, 1500 m, 22°38'S, 45°07'W	O. Mielke & M. Casagrande	AM021	DZUP 27453
Epicharis (Epicharana) flava Friese, 1900	Brazil, GO, Alto Paraíso de Goiás, 1 May 2010, 14º 19'S, 47º30'W, 1089 m	A. Aguiar	AM070	DZUP 27484
Epicharis (Epicharana) flava Friese, 1900	Brazil, Minas Gerais, Serranópolis de Minas, Jan. 2012	G. Melo	AM118	DZUP 27500
Epicharis (Epicharana) aff. *bova* Snelling, 1984	México, Chiapas, 8km SW de El Triunfo, 15 Aug. 2013, 16.1363°N, 91.8889°W, 1572 m	G. Melo & B. Rosa	AM172	DZUP 25646
Epicharis (Epicharana) elegans Smith, 1861	México, Chiapas, 8km SW de El Triunfo, 15 Aug. 2013, 16.1363°N, 91.8889°W, 1572 m.	G. Melo & B. Rosa	AM173	DZUP 25647
Epicharis (*Epicharana*) cf. *lindigi* Friese, 1899	Peru, Cusco, Quincemil, 1 Sep. 2012	B. Rosa	AM113	DZUP 27495
Epicharis (Epicharana) pygialis (Friese,	Brazil, AM, Presidente Figueiredo, AM 240	P. Grossi & D.	AM115	DZUP 27497

1900)	km 11 Pousada Bairro D'Água, LUZ, 23 Jul. 2008, 2º03'12"S, 59º56'14"W	Parizotto		
Epicharis (Epicharis) bicolor Smith, 1854	Brazil, MG, Serra do Salitre, RPPN Cachoeira do Campo, 1102 m, 19°09'30"S, 46°33'59"W, 1-5 Jan. 2009	A. Aguiar & A. Martins	AM022	DZUP 27454
Epicharis (Epicharis) bicolor Smith, 1854	Brazil, GO, Alto Paraíso de Goiás, 1 May 2010, 14°19'S, 47°30'W, 1089 m	A. Aguiar	AM029	UNB 5102
Epicharis (Epicharis) umbraculata (Fabricius, 1804)	Brazil, AM, Novo Airão, Pousada Bela Vista, 04 Jul. 2008, 36 m, 2º37’33”S/ 60º56’36”W	P. Grossi & D. Parizotto	AM198	DZUP 28419
Epicharis (Epicharitides) cockerelli Friese, 1900	Brazil, Paraná, Jaguariaíva, Parque Estadual do Cerrado, 19-21 Nov. 2009	E. Carneiro	AM024	DZUP 27456
Epicharis (Epicharitides) iheringi Friese, 1899	Brazil, GO, Cavalcante, 13°46'36"S, 47°24'42"W, 802 m, 03 Apr. 2010	A. Aguiar & A. Martins	AM030	UNB 5103
Epicharis (Epicharitides) minima (Friese, 1904)	Brazil, MT, 9 km a NW de Chapada dos Guimarães, PNCG, 15.411ºS 55.824ºW, 620 m, 15 Nov. 2013,	Melo, Luz & Williams	AM197	DZUP 28420
Epicharis (Epicharitides) luteocincta Moure & Seabra, 1959	Brazil, MT, 4 km a NW de Chapada dos Guimarães, 15.424ºS 55.770ºW, 690 m, 14 Nov. 2013	Melo, Luz & Williams	AM199	DZUP 28422
Epicharis (Epicharoides) picta (Smith, 1874)	Brazil, Paraná, Parque Estadual de Vila Velha, 28 Dec. 2007	G. Melo	AM017	DZUP 27449
Epicharis (Epicharoides) maculata Smith, 1874	Belize: Stann Creek/Dist. 4 kmW of/Middlesex, 1 May 2009	J.S. Ascher	AM105	AMNH 88353
Epicharis (Epicharoides) xanthogastra Moure & Seabra, 1959	Brazil, MT, 9 km a NW de Chapada dos Guimarães, PNCG, 15.411ºS 55.824ºW, 620 m, 15 Nov. 2013	Melo, Luz & Williams	AM196	DZUP 28421
Epicharis (Hoplepicharis) affinis Smith, 1874	Brazil, Amazonas, Manaus, Margem do Rio Negro, Praia do Tupé, 22 Oct. 2006	A. Aguiar	AM068	DZUP 27482
Epicharis (Hoplepicharis) affinis Smith,	Bolívia, Santa Cruz, Buena Vista, Hotel Fauna	G. Melo & A. Aguiar	AM116	DZUP 27498

1874	y Flora, 09. Feb. 2011, 409 m, 17º29'S 63º39'W			
Epicharis (Hoplepicharis) lunulata Mocsáry, 1898	Belize: Stann Creek/Dist. 4 km W of/Middlesex, 1 May 2009	J.S. Ascher	AM104	AMNH 178834
Epicharis (Parepicharis) zonata Smith, 1854	Brazil, MT, 4 km a NW de Chapada dos Guimarães, 15.424ºS 55.770ºW, 690 m, 14 Nov. 2013	Melo, Luz & Williams	AM200	DZUP 28423

1. Acronyms of entomological collections are: **AAC**: Andrea A. Cocucci, private collection, Córdoba, Argentina; AMNH: American Museum of Natural History; **BBSL**: Bee Biology and Systematic Laboratory, United States Department of Agriculture, Logan, Utah, United States; **CAS**: California Academy of Sciences, San Francisco, California, United States; **C.R.**: Claus Rasmussen Collection, Aarhus, Denmark; **DZUP**: Departamento de Zoologia da Universidade Federal do Paraná, Curitiba, Paraná, Brazil; **ECO-TAP**: Colección de insectos asociados a plantas cultivadas en la frontera sur, El Colegio de la Frontera Sur, Unidad Tapachula, Chiapas, Mexico; **UNB:** Colecao de insetos da Universidade de Brasilia, Brasilia, Distrito Federal, Brazil; **J.L, Neff**: Central Texas Melittological Institute, Austin, Texas, United States.

Table A2. Species for which sequences were downloaded from Genbank, with traditional taxon classification and collecting data information

a) Ingroup accessions

Tribe	Species	Collecting data
Apini	*Apis cerana* Fabricius, 1793	Japan, Kyoto
Apini	*Apis dorsata* Fabricius, 1793	Laos, Laksao
Apini	*Apis florea* Fabricius, 1787	Laos, Mahaxai
Bombini	*Bombus ardens* Smith, 1879	Japan, Kyoto
Bombini	*Bombus diversus* Smith, 1869	Japan, Kyoto
Bombini	*Bombus mendax* Gerstäcker, 1869	Italy: Monte Rosa
Centridini	*Centris (Paracentris) atripes* Mocsáry, 1899	USA: Arizona, Cochise Co., W. Turkey Creek
Centridini	*Centris (Centris) decolorata* Lepeletier, 1841	French Guiana: Cayenne, Cayenne. 7 Jul. 2006
Centridini	*Centris (Centris) varia* (Erichson, 1849)	Costa Rica
Centridini	*Centris (Melacentris) dimidiata* (Olivier, 1789)	French Guiana: Cayenne, Cayenne. 6 Jul. 2006
Centridini	*Centris (Trachina) longimana* Fabricius, 1804	French Guiana: Maripasoula, Saül. vil. 2 Jul. 2006
Centridini	*Centris (Paracentris) hoffmanseggiae* Cockerell, 1897	USA: California, Kern Co.,5mi S. Mojave. 13 Jun. 1999
Centridini	*Centris (Paracentris) cockerelli* Fox, 1899	USA: New York
Centridini	*Centris sp.* MP124	Colombia
Centridini	*Epicharis (Triepicharis) analis* Lepeletier, 1841	Paraguay: Paraguarí, Salto Cristal. 10 Feb. 2007
Centridini	*Epicharis* sp. 1 (JS 2010)	French Guiana: Kourou, Kourou. 11 Jul. 2006
Centridini	*Epicharis* sp. 2 (MP125)	Colombia
Euglossini	*Aglae caerulea* Lepeletier & Serville, 1825	Peru: Lagunas, Loreto
Euglossini	*Eufriesea surinamensis* (Linnaeus, 1758)	French Guiana: Kaw, Rt D6. 3o Nov. 2006
Euglossini	*Eufriesea pulchra* (Smith, 1854)	Panama
Euglossini	*Euglossa piliventris* Guérin-Méneville, 1845	French Guiana: Kaw, Rt D6. 30 Nov. 2006
Euglossini	*Euglossa imperialis* Cockerell, 1922	Panama
Euglossini	*Eulaema meriana* (Olivier, 1789)	French Guiana: Maripalousa, Saül. 4 Jul. 2006
Euglossini	*Exaerete frontalis* (Guérin-Méneville, 1845)	Panama
Meliponini	*Axestotrigona ferruginea* (Spinola, 1853)	South Africa: NP, 40km W. Sibasa. 20 Apr. 2002
Meliponini	*Cephalotrigona capitata* (Smith, 1854)	Costa Rica
Meliponini	*Hypotrigona gribodoi* (Magretti, 1884)	South Africa: Limpopo Prov., 27km E. Waterpoort. 7 Jan. 2004

Meliponini	*Meliponula bocandei* (Spinola, 1853)	Gabon
Meliponini	*Plebeina hildebrandti* (Friese, 1900)	South Africa: NP, 53 km S. Louis Trichardt. 16 Mar. 2002
Meliponini	*Scaptotrigona hellwegeri* (Friese, 1900)	Mexico: Estado de Jalisco, Carretera. 2.ix.2004
Meliponini	*Tetragonula carbonaria* (Smith, 1854)	Australia: NSW Windsor, 70 km N. 2 Dec.1999
c) Outgroup accessions		
Tribe	**Species**	**Collecting data**
Apinae		
Allodapini	*Braunsapis madecassella* Michener, 1977	Madagascar: 4km N. Tulear. 17 Oct. 2004
Allodapini	*Exoneura bicolor* Smith, 1854	Australia: VIC Flowerdale Forest. 20 Nov. 1999
Allodapini	*Macrogalea ellioti* (Saussure, 1890)	Madagascar: Ifaty, N. Tulear. 17 Oct. 2004
Ammobatini	*Pasites maculatus* Jurine, 1807	Romania: Histria. 6 Jul. 2000
Ammobatoidini	*Holcopasites arizonicus* (Linsley, 1942)	USA: Arizona, Cochise Co., 1 mi E. Douglas. 23 Dec. 2006
Ancylini	*Ancyla anatolica* Warncke, 1979	Turkey: Adana Prov., Serik. 20 Jun.2001
Anthophorini	*Anthophora urbana* Cresson, 1878	CA: Santa Clara Co., Del Puerto Cyn. 27 May 1999
Anthophorini	*Amegilla asserta* (Cockerell, 1926)	Australia: SA, 59km N. Cowell. 6 Jan. 999
Anthophorini	*Deltoptila aurulentocaudata* (Dours, 1869)	Mexico: Estado de Jalisco, Reserva Biosfera Sierra de Manantlan. 11 Sep. 2004
Anthophorini	*Habropoda laboriosa* (Fabricius, 1804)	USA: Florida, Alachua Co., Gainesville. 16 Apr. 2002
Anthophorini	*Pachymelus peringueyi* (Friese, 1911)	SOUTH AFRICA: NCP Dassiefontein, Kamieskroon, 16 Sep. 2001
Caenoprosopidini	*Caenoprosopina holmbergi* Roig-Alsina, 1987	Argentina: Salta Prov., Cachi. 4-8 Feb. 2006
Ceratinini	*Ceratina calcarata Robertson, 1900*	USA: New York, Tompkins Co., Ithaca. 4 Dec.1999
Ceratinini	*Ceratina (Crewella)* sp.	Paraguay: Cordillera, 6km SW Pirebebuy. 16 Jan. 2007

Ceratinini	*Ceratina (Simioceratina)* sp.	South Africa: NP, 29km NW. Waterpoort. 17 Apr.2002
Ceratinini	*Ceratina cyanea* (Kirby, 1802)	Czech Republic: Bestvina, Jul. 2000
Ctenoplectrini	*Ctenoplectra albolimbata* Magretti, 1895	SOUTH AFRICA: KZN, 20 km E Hluhluwe, 9-19 Mar. 2002
Ctenoplectrini	*Ctenoplectra bequaerti* Cockerell, 1930	Nigeria: Cross River State, Afi Mountain. 12 Mar.2006
Ctenoplectrini	*Ctenoplectrina* sp.	Nigeria: 2006
Emphorini	*Diadasia bituberculata* (Cresson, 1878)	CA: Contra Costa Co.Mitchell Cyn. 5 Jun. 1999
Emphorini	*Diadasina distincta* (Holmberg, 1903)	Paraguay: Boquerón, 2km NE. Filadélfia. 6 Feb. 2007
Emphorini	*Ancyloscelis* sp.	Paraguay: Cordillera, 6km SW Pirebebuy. 16.i.2007
Epeolini	*Triepeolus robustus* (Cresson, 1878)	USA: Arizona, Cochise Co, Chiricahua Monument. 14.ix.1999
Ericrocidini	*Ericrocis lata* (Cresson, 1878)	USA: Arizona, Cochise Co., 2mi N. Rodeo. 8.xi.1999
Ericrocidini	*Epiclopus gayi* Spinola, 1851	Chile: Curico prov., Laguna de Teno
Ericrocidini	*Mesonychium asteria* (Smith, 1854)	Paraguay: Boquerón, Fn. Toledo. 4.ii.2007
Eucerini	*Martinapis luteicornis* (Cockerell, 1896)	USA: Arizona, Cochise Co., 4mi E. Willcox. 25.iix.2001
Eucerini	*Xenoglossa angustior* Cockerell, 1899	USA: Arizona, Cochise Co., 2mi N. Portal. 11 Sep. 1999
Exomalopsini	*Anthophorula completa* (Cockerell, 1935)	USA: Arizona, Cochise Co., Comm. Rd. 12 Sep.1999
Iseopeolini	*Isepeolus atripilis* Roig-Alsina, 1991	Chile: Curico prov., Laguna de Teno
Iseopeolini	*Melectoides bellus* (Jörgensen, 1912)	Argentina: Salta Prov., Cachi. 12 Feb. 2004
Manueliini	*Manuelia gayatina* (Spinola, 1851)	Chile: Reg VIII, Entrepiernas, W. of Recinto. 8 Dec. 2004
Melectini	*Thyreus delumbatus* (Vachal, 1903)	South Africa: NP, 14km E. Vivo. 17 Apr. 2002

Melectini	*Xeromelecta californica* (Cresson, 1878)	USA: California, Santa Clara Co., Del Puerto Canyon. 27 May 1999
Melectini	*Zacosmia maculata* (Cresson, 1879)	USA: New Mexico, Grant Co., Hachita. 24 Sep.1999
Nomadini	*Nomada maculata* Cresson, 1863	USA: New York, Tompkins Co., Ithaca. 3 May 1999
Osirini	*Epeoloides pilosula* (Cresson, 1878)	USA: Connecticut, New London Co., Bozrah Rt 163. 22 Jun 2006
Osirini	*Parepeolus aterrimus* (Friese, 1906)	French Guiana: Kourou
Osirini	*Osiris* sp.	Peru: Puerto Maldonado. 1-3 Jan. 2007
Protepeolini	*Leiopodus abnormis* (Jörgensen, 1912)	Argentina: Salta Prov., Cachi. 4-8 Feb. 2006
Rhathymini	*Rhathymus unicolor* (Smith, 1854)	Paraguay:San Pedro, 30km S. Cororo. 27 Jan. 2007
Rhathymini	*Rhathymus* sp.	West Indies: Trinidad, Cauara Valley. 6-7 Feb. 2005
Rhathymini	*Nanorhathymus* sp.	West Indies: Trinidad, Cauara Valley. 17 Jul. 2003
Tapinotaspidini	*Arhysoceble picta* (Friese, 1899)	Paraguay: Paraguarí, Caballero. 21 Jan. 2007
Tapinotaspidini	*Caenonomada* sp.	Paraguay: Concepción, Vallemi. 1 Feb. 2007
Tetrapediini	*Coelioxoides waltheriae* Ducke, 1908	Argentina: Jujuy Prov., Libertador General San Martín. 2-3 Feb. 2006
Tetrapediini	*Tetrapedia maura* Cresson, 1878	Mexico: Estado de Jalisco, Reserva Biosfera Chamela-Cuixmala. 1 Sep. 2004
Tetrapediini	*Tetrapedia (Tetrapedia)* sp.	Argentina: Jujuy Prov., Libertador General San Martín. 8-10 Feb. 2004,
Xylocopini	*Xylocopa iris* (Christ, 1791)	Turkey: Izmir Prov., Selcuk. 24 Jul. 2006
Xylocopini	*Xylocopa fimbriata* Fabricius, 1804	French Guiana: Cayenne, Cayenne. 6 Jul. 2006
Xylocopini	*Xylocopa muscaria* (Fabricius, 1775)	French Guiana: Kourou, Kourou. 11 Jul. 2006
Xylocopini	*Xylocopa pubescens* Spinola, 1838	Tunisia: Blidette vill. 25-27 Feb. 2006

Xylocopini	*Xylocopa tabaniformis* Smith, 1854	USA: California, Santa Clara Co., Mt. Hamilton. 27 May 1999
Xylocopini	*Xylocopa violacea* (Linnaeus, 1758)	Turkey: Adana Prov., Jun. 2001
Megachilinae		
Fideliini	*Fidelia major* (Friese, 1911)	South Africa: WCP, 5km N. Clanwilliam. 20 Sep. 2001
Fideliini	*Neofidelia profuga* Moure and Michener, 1955	Chile: Atacama Prov., Inca-havas 5 km N. 3 Oct.1997
Pararhophitini	*Pararhophites quadratus* (Friese, 1898)	Tunisia: Blidette vill. 25-27 Mar. 2006
Anthidiini	*Anthidiellum notatum* (Latreille, 1809)	USA: Arizona, Cochise Co., Comm. Rd. 12 Sep. 1999
Anthidiini	*Anthidium porterae* Cockerell, 1900	USA: New Mexico, Hidalgo Co. 20 mi S. Animas. 17 Sep. 1999
Lithurgini	*Lithurgus echinocacti* Cockerell, 1898	USA: Arizona, Pima Co., Tucson. 4 Sep. 2000
Lithurgini	*Trichothurgus herbsti* (Friese, 1905)	Chile: Region VIII, Las Trancas, 78 km E. Chillan. 12 Dec. 2003
Osmiini	*Afroheriades hyalinus* Griswold & Gonzalez 2011	South Africa: WCP, 7 km W. Nieuwoudtville. 9 Nov. 2002

Table A3. GenBank accession numbers for all newly generated sequences. Voucher information is provided in Table S1.

a) Species in the tribe Centridini newly sequenced for this study.

Species	**28S**	**Poll II**	**Opsin**	**EF-1α**
C. (Aphemisia) lilacina Cockerell, 1919	KM265396	KM257741	KM269856	KM269501
Centris (Aphemisia) mocsary Friese, 1899	KM265379	KM257742	KM269839	KM269540
Centris (Aphemisia) plumipes Smith, 1874	KM265395	KM257743	KM269860	KM269557
Centris (*Aphemisia*) *agilis* Smith, 1874	KM265430	KM257740	KM269810	KM269551
Centris (*Aphemisia*) *sericea* Friese, 1899	KM265366	KM257744	KM269852	KM269504
Centris (Centris) varia (Erichson, 1849)	KM265378	KM257770	-	KM269509
Centris (Centris) aenea Lepeletier, 1841		KM257767	KM269832	KM269535
Centris (Centris) haemorrhoidalis (Fabricius, 1775)	KM265429	KM257768	KM269850	KM269506
Centris (Centris) spilopoda Moure, 1969	KM265439	KM257769	-	KM269512
Centris (Hemisiella) vittata Lepeletier, 1841	KM265372	KM257749		KM269491
Centris (H.) tarsata Smith, 1874	KM265418	KM257746	KM269841	KM269521
Centris (H.) trigonoides Lepeletier, 1841	KM265368	KM257748	KM269848	KM269531
Centris (H.) nitida Smith, 1874	KM265400	KM257745	KM269829	KM269558
Centris (H.) transversa Pérez, 1905	KM265406	KM257747	KM269853	KM269525
Centris (Heterocentris) analis (Fabricius, 1804)	KM265436	KM257784	KM269849	KM269529
Centris (H.) difformis Smith, 1854	KM265423	KM257785	KM269831	KM269527
Centris (H.) labrosa Friese, 1899	KM265402	KM257786	KM269844	KM269532
Centris (Melacentris) violacea Lepeletier, 1841	KM265422	KM257796	KM269835	KM269554
Centris (M.) dorsata Lepeletier, 1841	KM265375	KM257792	KM269862	KM269553
Centris (M.) rhodoprocta Moure & Seabra, 1960	KM265382	KM257794	-	KM269492
Centris (M.) rufohirta Friese, 1900	KM265386	KM257795	KM269847	
Centris (M.) braccata Packard, 1869	KM265417	KM257790	-	-
Centris (M.) conspersa Mocsáry, 1899	KM265411	KM257791	-	KM269489
Centris (M.) xanthocnemis (Perty, 1833)	KM265397	KM257797	-	KM269537
Centris (M.) aff. *dorsata* Lepeletier, 1841		KM257789	KM269855	KM269507
Centris (*M.*) sp.	KM265371	-	KM269830	KM269520
Centris (*M.*) *gelida* Snelling, 1984	KM265380	KM257793	KM269837	KM269517
Centris (Paracentris) agameta Snelling, 1974	KM265438	KM257799	KM269807	KM269523
Centris (P.) albiceps Friese, 1899	KM265428	KM257800	KM269828	KM269556
Centris (P.) anomala Snelling, 1966	KM265424	KM257801	KM269821	KM269526
Centris (P.) nigrocaerulea Smith, 1874	KM265414	KM257808		KM269549
Centris (P.) zacateca Snelling, 1966	KM265401	KM257813	KM269861	KM269496
Centris (*P.*) *cockerelli* Fox, 1899	KM265384	-	-	KM269545
Centris (P.) hyptidis Ducke, 1908	KM265416	KM257804	KM269846	KM269519
Centris (P.) xanthomelaena Moure & Castro, 2001	KM265394	KM257812	KM269859	KM269552
Centris (P.) nigerrima (Spinola, 1855)	KM265389	KM257807	-	KM269499
Centris (*P.*) *burgdorfi* Friese, 1900	KM265373	KM257802	KM269818	KM269511
Centris (P.) klugii Friese, 1899	KM265407	KM257805	KM269816	KM269485
Centris (P.) thelyopsis Vivallo & Melo, 2009	KM265410	KM257810	KM269833	KM269543
Centris (P.) mexicana Smith, 1854	KM265381	KM257806		KM269498
Centris (P.) pallida Fox, 1899	KM265437	KM257809		KM269555

Centris (P.) caesalpinae Cockerell, 1897	KM265363	KM257803	KM269851	KM269518
Centris (P.) lanosa Cresson, 1872	KM265388	-	-	-
Centris (P.) tricolor Friese, 1899	KM265421	KM257811	KM269823	KM269516
Centris (P.) aff. *neffi* Moure, 2000	KM265425	KM257798	KM269820	KM269486
Centris (Penthemisia) chilensis (Spinola, 1851)	KM265377	-	-	KM269488
Centris (P.) chilensis (Spinola, 1851)	-	KM257751	KM269803	-
Centris (P.) cfr. *chilensis* (Spinola, 1851)	KM265413	-	KM269812	-
Centris (P.) tamarugalis Toro & Chiappa, 1989	KM265365	KM257752	KM269826	KM269494
Centris (P.) buchholzi Herbst, 1918	KM265399	KM257750	-	KM269524
Centris (Ptilocentris) chlorura Cockerell, 1919	KM265369	KM257815	KM269854	KM269533
Centris (Ptilotopus) scopipes Friese, 1899	KM265364	KM257755	KM269857	KM269487
Centris (P.) decipiens Moure & Seabra, 1960	KM265427	KM257753	KM269845	KM269503
Centris (P.) moerens (Perty, 1833)	KM265383	KM257754	KM269834	KM269497
Centris (P.) sponsa Smith, 1854	KM265440	KM257756	KM269858	KM269539
Centris (Trachina) rupestris Azevedo & Silveira, 2005	KM265412	KM257759	KM269843	KM269505
Centris (T.) similis(Fabricius, 1804)	KM265374	KM257760	-	KM269541
Centris (T.) fuscata Lepeletier, 1841	KM265420	KM257758	KM269838	KM269510
Centris (T.) aff. ocellaris (Ducke, 1906)	KM265387	KM257757	KM269836	KM269550
Centris (Wagenknechtia) muralis Burmeister, 1876	KM265385	KM257762	KM269842	KM269514
Centris (W.) rhodophtalma Pérez, 1911	KM265392	KM257763	KM269840	KM269548
Centris (W.) cineraria Smith, 1854		KM257761	-	KM269508
Centris (Xanthemisia) bicolor Lepeletier, 1841	KM265431	KM257764	KM269811	KM269547
Centris (X.) cf. *carolae* Snelling, 1866	KM265435	KM257765	KM269815	KM269530
Centris (X.) lutea Friese, 1899	KM265409	KM257766	-	KM269544
Epicharis (A.) dejeanii Lepeletier, 1841	KM265432	KM257739	KM269819	KM269536
Epicharis (C.) morio Friese, 1924	KM265376	KM257771	KM269824	KM269515
Epicharis (E.) flava Friese, 1900	-	-	KM269827	KM269538
Epicharis (E.) flava Friese, 1900	KM265390	KM257774		-
Epicharis (E.) aff. bova Snelling, 1984	KM265415	KM257772	KM269817	KM269528
Epicharis (E.) elegans Smith, 1861	KM265391	KM257773	KM269799	KM269500
Epicharis (*E.*) cf. *lindigi* Friese, 1899	KM265441	-	-	KM269522
Epicharis (E.) pygialis (Friese, 1900)	KM265405	KM257775	KM269822	KM269495
Epicharis (E.) bicolor Smith, 1854	-	-	-	KM269493
Epicharis (E.) bicolor Smith, 1854	KM265404	KM257776	KM269808	-
Epicharis (E.) umbraculata (Fabricius, 1804)	KM265393	KM257777		KM269484
Epicharis (E.) cockerelli Friese, 1900	KM265408	KM257778	KM269802	KM269545
Epicharis (E.) iheringi Friese, 1899	KM265419	KM257779	KM269825	KM269534
Epicharis(E.) minima (Friese, 1904)	KM265367	KM257781	KM269800	KM269502
Epicharis (E.) luteocincta Moure & Seabra, 1959	KM265403	KM257780	KM269805	
Epicharis (E.) picta (Smith, 1874)	KM265434	KM257782	KM269806	KM269542
Epicharis (Epicharoides) maculata Smith, 1874	KM265426	-	KM269809	-
Epicharis (E.) xanthogastra Moure & Seabra, 1959	KM265433	KM257783	KM269804	-
Epicharis (H.) affinis Smith, 1874		KM257787		KM269490
Epicharis (H.) affinis Smith, 1874	KM265442		KM269801	
Epicharis (H.) lunulata Mocsáry, 1898	KM265370	KM257788	KM269813	KM269513
Epicharis (P.) zonata Smith, 1854	KM265398	KM257814	KM269814	KM269546

b) Ingroup taxa from Genbank

Species	**28S**	**Poll II**	**Opsin**	**EF-1α**
Apis cerana Fabricius, 1793	HM750244	EU184733	EU184839	EU184774
Apis dorsata Fabricius, 1793	HM750243	EU184732	AY267162	AY267146
Apis florea Fabricius, 1787	HM750242	EU184731	EU184838	EU184773
Bombus ardens Smith, 1879	HM750237	EU184724	AF493031	AF492964
Bombus diversus Smith, 1869	HM750236	EU184725	AF493028	AF492961
Bombus mendax Gerstäcker, 1869	HM750235	EU184726	AF493024	AF492957
Centris (Paracentris) atripes Mocsáry, 1899	GU244764	GU245363	GU245239	-
Centris (Centris) decolorata Lepeletier, 1841	GU244763	GU245362	GU245245	GU244924
Centris (Centris) varia (Erichson, 1849)	-	-	AF181577	-
Centris (Melacentris) dimidiata (Olivier, 1789)	GU244761	GU245360	GU245243	GU244922
Centris (Trachina) longimana Fabricius, 1804	GU244762	GU245361	GU245244	GU244923
Centris (Paracentris) hoffmanseggiae Cockerell, 1897	GU244765	GU245364	AF344590	GU244918
Centris (Paracentris) cockerelli Fox, 1899	-	EU184734	AY267164	AY267148
Centris sp. MP124	-	EU162869	-	EU163200
Epicharis (Triepicharis) analis Lepeletier, 1841	GU244759	GU245358	GU245241	GU244920
Epicharis sp. 1 (JS 2010)	GU244760	GU245359	GU245242	GU244921
Epicharis sp. 2 (MP125)	-	EU162870	-	EU163201
Aglae caerulea Lepeletier & Serville, 1825	GU244797	GU245396	GU245271	-
Eufriesea surinamensis (Linnaeus, 1758)	GU244798	GU245397	GU245272	GU244947
Eufriesea pulchra (Smith, 1854)	HM750234	EU184723	EU184834	EU184769
Euglossa piliventris Guérin-Méneville, 1845	GU244796	-	GU245270	GU244946
Euglossa imperialis Cockerell, 1922	HM750232	EU184721	AY267160	AY267144
Eulaema meriana (Olivier, 1789)	GU244795	GU245394	GU245269	GU244945
Exaerete frontalis (Guérin-Méneville, 1845)	HM750233	EU184722	AY267159	AY267143
Axestotrigona ferruginea (Spinola, 1853)	GU244812	AY945192	GU245281	GU244958
Cephalotrigona capitata (Smith, 1854)	HM750239	EU184728	EU184836	EU184771
Hypotrigona gribodoi (Magretti, 1884)	GU244811	AY945189	GU245280	GU244957
Meliponula bocandei (Spinola, 1853)	HM750240	EU184729	AY267161	AY267145
Plebeina hildebrandti (Friese, 1900)	GU244816	GU245413	GU245284	GU244962

Scaptotrigona hellwegeri (Friese, 1900)	GU244817	GU245414	GU245285	GU244963
Tetragonula carbonaria (Smith, 1854)	GU244814	GU245411	GU245282	GU244960

c) Outgroup accessions from Genbank

Species	**28S**	**Poll II**	**Opsin**	**EF-1α**
Apinae				
Braunsapis madecassella Michener, 1977	GU244893	GU245490	GU245231	GU245038
Exoneura bicolor Smith, 1854	GU244896	GU245493	GU245337	GU245041
Macrogalea ellioti (Saussure, 1890)	GU244895	GU245492	GU245336	GU245040
Pasites maculatus Jurine, 1807	GU244867	GU245464	HM211842	GU245035
Holcopasites arizonicus (Linsley, 1942)	GU244869	GU245466	GU245319	GU245013
Ancyla anatolica Warncke, 1979	GU244753	GU245352	GU245235	GU244913
Anthophora urbana Cresson, 1878	GU244755	GU245354	AF344585	GU244915
Amegilla asserta (Cockerell, 1926)	GU244756	GU245355	GU245237	GU244916
Deltoptila aurulentocaudata (Dours, 1869)	GU244757	GU245356	GU245238	GU244917
Habropoda laboriosa (Fabricius, 1804)	GU244754	GU245353	GU245236	GU244914
Pachymelus peringueyi (Friese, 1911)	AY654544	AY945151	DQ116678	AY585114
Caenoprosopina holmbergi Roig-Alsina, 1987	GU244879	GU245476	GU245325	GU244983
Ceratina calcarata Robertson, 1900	AY654535	AY945099	-	AY585108
Ceratina (Crewella) sp.	GU244897	GU245494	GU245338	GU245042
Ceratina (Simioceratina) sp.	GU244898	GU245495	GU245339	GU245043
Ceratina cyanea (Kirby, 1802)	GU244901	GU245498	HM211848	GU244984
Ctenoplectra albolimbata Magretti, 1895	AY654538	AY945111	DQ116677	AY585118
Ctenoplectra bequaerti Cockerell, 1930	GU244767	GU245366	GU245247	GU244926
Ctenoplectrina sp.	GU244766	GU245365	GU245246	GU244925
Diadasia bituberculata (Cresson, 1878)	GU244768	GU245367	AF344594	GU244927
Diadasina distincta (Holmberg, 1903)	GU244770	GU245369	GU245248	GU244929
Ancyloscelis sp.	GU244774	GU245373	GU245252	GU244933
Triepeolus robustus (Cresson, 1878)	AY654547	AY945170	AF344634	GU245023
Ericrocis lata (Cresson, 1878)	GU244777	GU245376	GU245255	GU244936
Epiclopus gayi Spinola, 1851	GU244776	GU245375	GU245254	GU244935
Mesonychium asteria (Smith, 1854)	GU244778	GU245377	GU245256	GU244937
Martinapis luteicornis (Cockerell, 1896)	DQ072147	DQ069333	-	-

Xenoglossa angustior Cockerell, 1899	GU244787	GU245386	-	-
Anthophorula completa (Cockerell, 1935)	GU244799	GU245398	AF344622	GU244948
Isepeolus atripilis Roig-Alsina, 1991	GU244804	GU245403	GU245276	GU244952
Melectoides bellus (Jörgensen, 1912)	GU244807	GU245406	HM211836	GU244999
Manuelia gayatina (Spinola, 1851)	GU244902	GU245499	GU245342	GU245046
Thyreus delumbatus (Vachal, 1903)	AY654546	AY945169	DQ116679	AY585119
Xeromelecta californica (Cresson, 1878)	GU244808	GU245407	AF344613	GU244955
Zacosmia maculata (Cresson, 1879)	AY654548	AY945176	AF344637	AY585117
Nomada maculata Cresson, 1863	GU244890	GU245487	AF344609	GU245030
Epeoloides pilosula (Cresson, 1878)	GU244819	GU245416	GU245287	GU244966
Parepeolus aterrimus (Friese, 1906)	GU244820	GU245417	GU245288	GU244967
Osiris sp.	GU244822	GU245419	HM211839	GU245033
Leiopodus abnormis (Jörgensen, 1912)	GU244824	GU245421	GU245290	GU244969
Rhathymus unicolor (Smith, 1854)	GU244828	GU245425	GU245294	GU244973
Rhathymus sp.	GU244826	GU245423	GU245292	GU244971
Nanorhathymus sp.	GU244827	GU245424	GU245293	GU244972
Arhysoceble picta (Friese, 1899)	GU244831	GU245428	GU245297	GU244976
Caenonomada sp.	GU244829	GU245426	GU245295	GU244974
Coelioxoides waltheriae Ducke, 1908	GU244838	GU245435	GU245303	-
Tetrapedia maura Cresson, 1878	GU244834	GU245431	GU245300	GU244979
Tetrapedia (Tetrapedia) sp.	GU244837	GU245434	HM211840	-
Xylocopa iris (Christ, 1791)	GU244906	GU245503	GU245345	GU245050
Xylocopa fimbriata Fabricius, 1804	GU244910	GU245507	GU245349	GU245053
Xylocopa muscaria (Fabricius, 1775)	GU244905	GU245502	GU245344	GU245049
Xylocopa pubescens Spinola, 1838	GU244908	GU245505	GU245347	GU245052
Xylocopa tabaniformis Smith, 1854	GU244904	GU245501	AF344614	GU245048
Xylocopa violacea (Linnaeus, 1758)	GU244909	GU245506	GU245348	GU245055
Megachilinae				
Fidelia major (Friese, 1911)	AY654539	AY945119	EU851628	-
Neofidelia profuga Moure and Michener, 1955	GU244840	GU245437	GU245305	GU244990
Pararhophites quadratus (Friese, 1898)	GU244841	GU245438	GU245306	GU245034
Anthidiellum notatum (Latreille, 1809)	GU244845	GU245442	AF344617	GU244995
Anthidium porterae Cockerell, 1900	GU244846	GU245443	AF344619	GU244996
Lithurgus echinocacti Cockerell, 1898	AY654541	AY945136	DQ116702	-

Trichothurgus herbsti (Friese, 1905)	GU244848	GU245445	GU245310	GU245000
Afroheriades hyalinus Griswold & Gonzalez 2011	GU244860	GU245457	GU245316	GU245007

Table A4. PCR primer sequences used in this study and PCR conditions (at bottom of table)

DNA Region	Base pairs	Primers names	Primers Sequences	Reference
28S rDNA[1]	**1200**	D2-3665F (Bel28S-For)	5'-AGAGAGAGTTCAAGAGTACGTG-3'	(1)
		D3-4283R (Mar28S-Rev)	5'-TAGTTCACCATCTTTCGGGTCCC-3'	(2)
		D3-4048F (28SD4For)	5'-CCCGTCTTGAAACACGGACCAAGG-3'	(3)
		D5-4749R (28SD4Rev)	5'-GTTACACACTCCTTAGCGGA-3'	(3)
RNA polymerase II[2]	**700**	polfor2a	5´-AAYAARCCVGTYATGGGTATTGTRCA-3´	(3)
		polrev2a	5´-GAAARATCTTYTGYACGTTGGADATC-3´	(3)
		RNApolrev	5'-TCGTCCGCAGACCTCGTGTCC-3'	This work
		RNApolfor	5'-AGAACACGGAGAACTCGTTATGG-3'	This work
LW-Rhodopsin[3]	**800**	OpsinFor	5'-AATTGCTATTAYGARACNTGGGT-3'	(2)
		OpsinRev	5'-ATATGGAGTCCANGCCATRAACCA-3'	(2)
		OpsinFor4	5'-GAGAARAAYATGCGBGARCAAGC-3'	(4)
		Opsin-61F	5'-TCCTTGTTCGGATGTGGCTC-3'	This work
		Opsin-620R	5'-TGCCAATTTACACTCAGCAC-3'	This work
EF-1α (F2 copy)[4]	**1100**	HaF2For1	5'- GGYAAAGGWTCCTTCAARTAGC-3'	(5)
		F2Rev1	5'- ATCAGCAGCACCTTTAGGTGG-3'	(5)
		EFfor1	5'-GGTACTGGCGAGTTTGAAGC-3'	This work
		EFFor2	5'-ACAAGACCCACAGACAAGGCTC-3'	This work
		EFRev1	5'-TGGCACAAATGCAACTGCAGC-3'	This work
		EFRev2	5'-CGGAGAGCCTTGTCTGTGGGTC-3'	This work

1. PCR conditions: **Bel28SFor/Mar28SRev**: 94°C for 1 min, 54-65°C for 1 min, 72°C for 1 min (35 cycles). **28SD4For/Rev**: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min.
2. PCR conditions: HaF2For1/F2Rev1: 94°C for 1 min, 54°C for 1 min, 72°C for 1.5 min (35 cycles). **HaF2for1/EFRev1**: 94°C for 1 min, 52-54°C for 1 min, 68°C for 1,5 min (39 cycles). **EFFor1/EFRev2**: 94°C for 1 min, 52-54°C for 1 min, 68°C for 1,5 min (39 cycles). **EFFor2/F2Rev1**: 94°C for 1 min, 52-54°C for 1 min, 68°C for 1,5 min (39 cycles).
3. PCR conditions: OpsinFor/OpsinRev: 94°C for 1 min, 52°C for 1 min, 72°C for 1 min (35 cycles); **OpsinFor4/OpsinRev**: 94°C for 1 min, 52°C for 1 min, 68°C for 1 min (39 cycles). **Opsin-61F/Opsin620R**: 94°C for 1 min, 50°C for 1 min, 68°C for 1 min (39 cycles).
4. PCR conditions: Polfor2a/polrev2a: 94° for 1 min, 52°C for 1 min, 72°C for 1 min. **Polfor2a/RNApolrev**: 94°C for 1 min, 52°C for 1 min, 68°C for 1 min (39 cycles). **RNApolfor/polrev2a**: 94°C for 1 min, 52°C for 1 min, 68°C for 1 min (39 cycles).

Table A5. Fossils used as calibrations or for cross validation with their assignment, stratum, age priors and justification.

Calibration fossils (node)	**Species name**	**Stratum and age priors (Ma)**	**Justification**
Crown of *Apis cerana* + *Apis dorsata*	*Apis lithohermaea* Engel, 2006	Middle Miocene, Langhian (?) (13.8 - 16 Mya, age of Langhian), Chôjabaru Formation BEAST: lognormal (Mean: 1.26; Stdev: 0.8; offset 15)	*Apis lithohermaea* was found in the Chôbaru Formation, Japan, from the middle Miocene, possibly Langhian age (6). This is the first fossil assigned to the crown-group of *Apis*. By its relatively big body size, Engel (6) placed this fossil in the *Apis dorsta* group. Based on Engel's assignment, we placed this fossil at the node uniting *A. dorsata* and *A. cerana*.
Sister to *Hypotrigona*	*Kelneriapis eocenica* (Kelner-Pillault, 1970)	Eocene, Lutetian (44 ±1.1 Mya), Baltic amber BEAST: lognormal (Mean: 1.67; Stdev: 0.5; offset 45)	This stingless bee fossil from the Baltic Amber was first assigned to an extant genus, *Hypotrigona*, the suspected sister to *Kelneriapis* (7). *Kelneriapis* along with the stingless bee fossils *Liotrigonopsis* are more related to Old World genera (7). Therefore, in our analyses we placed *K. eocenica* as sister to the clade formed by the African species.
Cross validation (node)			
Crown of *Apis*	*Apis henshawi* Cockerell 1907	Oligocene (33-23- age of the oligocene), Rott	The oldest honeybee fossil is from Oligocene deposits of Rott, Germany (8). The phylogenetic position of this and other honeybee fossils is uncertain and only *A. lithohermaea* can be assigned to the monophyletic group consisting of the extant species (6). We used *A. henshawi* as providing a minimum age for the *Apis* crown.
Crown of Allodapini	Boreallodapini	Eocene, Lutetian (44 ±1.1 Mya), Baltic amber	Boreallodapini is a bee tribe from the Baltic amber, where its single genus *Boreallodape* comprises three species (7). Engel (7) argued that Boreallodapini is closely related to Ceratinini and Allodapini, all three sharing at least three morphological characters, indicating that Ceratinini is sister to Allodapini+Boreallodapini. For this reason we placed this fossil at

			the crown of Ceratinini + Allodapini.
Stem of Meliponini	*Cretotrigona prisca (*Michener & Grimaldi1998*)*	Late Cretaceous, Maastrichtian (66-72.1); New Jersey Amber;	*Cretotrigona prisca* was found in the amber of New Jersey and was first assigned to the genus *Trigona* (9). Later, it was transferred to a new genus, *Cretotrigona*, and suggested to be the sister to the Old World genus *Dactylurina* (10). In spite of the uncertainty in the phylogenetic position of *Cretotrigona*, it has many morphological characters of the modern stingless bees. However, its age fits only with it having been an extinct lineage of Meliponini.
Crown of Anthophorini	*Paleohabropoda oudardi* Michez & Rasmont 2009	Paleocene, 60 Mya spongo-diatomitic volcanic paleo-lake (maar) deposit, Menat, Puy-de-Dôme, France	This fossil was found in the French Paleocene of Menat, possibly at an age of 60 Mya and (11) assigned it to the tribe Anthophorini by the ratio of first and second submarginal cells. However, they could not include this fossil in any of the extant anthophorine genera. According to the cladistic analysis, *Paleohabropoda* is closely related to the extant genera *Habrophorula* and *Elaphropoda* (11), which lead us to place this fossil as sister-group to all Anthophorini representatives in our sampling.

Table A6. Estimated ages for the higher clades of long-tongued bees from the present study, comparing with ages obtained by other studies, when the refereed clade was recovered.

	Ages (Ma) of the crown					
Clade	**Present paper (95% HPD)**	(12)	(13)	(14)	(15)	(16)
Long-tonged bees	117 (102-138)	115	115-120	-	140	-
Megachilinae sensu lato	94 (70-123)	90-95	-	140-145	-	-
Apinae sensu lato	108 (99-117)	100	110	-	-	-
Cleptoclade+Anthophorini	99 (87-111)	95	105	-	111 (95-129)	-
Xylocopinae+Eucerini line	96 (85-107)	95	100-105	-	-	-
Apine clade	91 (79-103)	85-90	95-100	-	-	-
Corbiculates	77 (66-89)	80	80-85	-	-	-
Apini	22 (17-29)	20-25	20-25	-	-	-
Euglossini	26 (17-38)	25-30	25-30	34-38	-	-
Bombini	28 (14-46)	15-20	20-25	-	-	25.4-47.3
Meliponini	51 (48-56)	50-55	45-50	-	-	-
Centris+Epicharis	-	70-75	-	-	-	-
Centris	46 (36-59)	-	35-40	-	-	-
Epicharis	28 (19-40)	-	30-35	-	-	-

Table A7. Oil-collecting behavior in the females: presence or absence. Genera abbreviations: *C.*: *Centris*, *E.*: *Epicharis*. Subgenera abbreviations: (*A.*): *Aphemisia*; (*C.*): *Centris*; (*He.*): *Hemisiella*; (*Ht.*): *Heterocentris*; (*M.*): *Melacentris*; (*Pa.*): *Paracentris*; (*Pe.*): *Penthemisia*; (*Pc.*) *Ptilocentris*; (*Pt.*): *Ptilotopus*; (*T.*): *Trachina*; (*W.*): *Wagenknechtia*; (*X.*): *Xanthemisia*; (*An.*): *Anepicharis*; (*Cy.*): *Cyphepicharis*; (*Ea.*): *Epicharana*; (*Ep.*) *Epicharis*; (*Et.*): *Epicharitides*; (*Eo.*): *Epicharoides*; (*Ho.*): *Hoplepicharis*; (*Pr.*): *Parepicharis*; (*Te.*): *Triepicharis*.

Species	**Oil-collecting apparatus**
Aglae caerulea	absent
Apis cerana	absent
Apis dorsata	absent
Apis florea	absent
Axestotrigona ferruginea	absent
Bombus ardens	absent
Bombus diversus	absent
Bombus mendax	absent
C. (*A.*) *agilis*	four-legged
C. (A.) lilacina	four-legged
C. (A.) mocsary	four-legged
C. (A.) plumipes	four-legged
C. (*A.*) *sericea*	four-legged
C. (C.) aenea	four-legged
C. (C.) decolorata	four-legged
C. (C.) haemorrhoidalis	four-legged
C. (C.) spilopoda	four-legged
C. (C.) varia	four-legged
C. (He.) nitida	four-legged
C. (He.) tarsata	four-legged
C. (He.) transversa	four-legged
C. (He.) trigonoides	four-legged
C. (He.) vittata	four-legged
C. (Ht.) analis	four-legged
C. (Ht.) difformis	four-legged
C. (Ht.) labrosa	four-legged
C. (M.) aff. *dorsata*	four-legged
C. (M.) braccata	four-legged
C. (M.) conspersa	four-legged
C. (M.) dimidiata	four-legged
C. (M.) dorsata	four-legged
C. (M.) gelida	four-legged
C. (M.) rhodoprocta	four-legged
C. (M.) rufohirta	four-legged
C. (*M.*) sp.	four-legged
C. (M.) violacea	four-legged
C. (M.) xanthocnemis	four-legged
C. (Pa.) aff. *neffi*	four-legged
C. (Pa.) agameta	four-legged

C. (Pa.) albiceps	four-legged
C. (Pa.) anomala	absent
C. (Pa.) atripes	four-legged
C. (*Pa.*) *burgdorfi*	four-legged
C. (Pa.) caesalpinae	four-legged
C. (Pa.) cockerelli	four-legged
C. (Pa.) hoffmanseggiae	absent
C. (Pa.) hyptidis	two-legged
C. (Pa.) klugii	four-legged
C. (Pa.) lanosa	four-legged
C. (Pa.) mexicana	four-legged
C. (Pa.) nigerrima	four-legged
C. (Pa.) nigrocaerulea	four-legged
C. (Pa.) pallida	absent
C. (Pa.) thelyopsis	two-legged
C. (Pa.) tricolor	four-legged
C. (Pa.) xanthomelaena	four-legged
C. (Pa.) zacateca	four-legged
C. (Pc.) chlorura	four-legged
C. (Pe.) buchholzi	four-legged
C. (Pe.) cf. *chilensis*	four-legged
C. (Pe.) chilensis	four-legged
C. (Pe.) tamarugalis	absent
C. (Pt.) decipiens	four-legged
C. (Pt.) moerens	four-legged
C. (Pt.) scopipes	four-legged
C. (Pt.) sponsa	four-legged
C. (T.) aff. *ocellaris*	four-legged
C. (T.) fuscata	four-legged
C. (T.) longimana	four-legged
C. (T.) rupestris	four-legged
C. (T.) similis	four-legged
C. (W.) cineraria	two-legged
*C. (W.) muralis**	absent
C. (W.) rhodophthalma	four-legged
C. (X.) bicolor	four-legged
C. (X.) cf. *carolae*	four-legged
C. (X.) lutea Friese, 1899	four-legged
C. sp.	four-legged
Cephalotrigona capitata	absent
Epicharis (Ep.) bicolor	four-legged
Epicharis (An.) dejeanii	four-legged
Epicharis (Cy.) morio	four-legged
Epicharis (Ea.) aff. *bova*	four-legged
Epicharis (*Ea.*) cf. *lindigi*	four-legged
Epicharis (Ea.) elegans	four-legged
Epicharis (Ea.) flava	four-legged

Epicharis (Ea.) pygialis	four-legged
Epicharis (Eo) maculata	four-legged
Epicharis (Eo.) picta	four-legged
Epicharis (Eo.) xanthogastra	four-legged
Epicharis (Ep.) umbraculata	four-legged
Epicharis (Et.) cockerelli	four-legged
Epicharis (Et.) iheringi	four-legged
Epicharis (Et.) luteocincta	four-legged
Epicharis (Et.) minima	four-legged
Epicharis (Ho.) affinis	four-legged
Epicharis (Ho.) lunulata	four-legged
Epicharis (Pr.) zonata	four-legged
Epicharis (Te.) analis	four-legged
Epicharis sp.2	four-legged
Epicharis sp.1	four-legged
Eufriesea pulchra	absent
Eufriesea surinamensis	absent
Euglossa imperialis	absent
Euglossa piliventris	absent
Eulaema meriana	absent
Exaerete frontalis	absent
Hypotrigona gribodoi	absent
Meliponula bocandei	absent
Plebeina hildebrandti	absent
Scaptotrigona hellwegeri	absent
Tetragonula carbonaria	absent

*oil collecting structure present, but they never were observed collecting oil (17)

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CAPÍTULO 2

SYSTEMATICS OF THE OIL BEES *CENTRIS* AND *EPICHARIS* BASED ON THE PHYLOGENY OF THE SUBFAMILY APINAE (HYMENOPTERA)

SYSTEMATICS OF THE OIL BEES *CENTRIS* AND *EPICHARIS* BASED ON THE PHYLOGENY OF THE SUBFAMILY APINAE (HYMENOPTERA)

ABSTRACT

Bees are economically and ecologically important group of animals by pollinating most of flowering plants, a 120 Ma old association. Bee systematics has undergone great progress in the last years by increasing use of nucleotide data and molecular based methods. The present work assessed the systematics of two solitary Neotropical groups of oil-collecting bees, *Centris* and *Epicharis*, traditionally classified in one tribe, Centridini. Molecular phylogenies for the subfamily Apinae indicated the paraphyly of this tribe and the sister relationship of *Centris* with the corbiculate bees. Each of these genera is strongly supported as monophyletic, but their inner phylogenetic relationships partially disagree with the taxonomic classification. *Centris* has three main clades, the groups *Centris*, *Trachina* and *Melacentris* already recovered by previous morphology based phylogenetic studies. On the twelve subgenera traditionally proposed for the genus *Centris*, only *Paracentris* and *Melacentris* are paraphyletic. In *Paracentris* is suggested the stronger taxonomic rearrangements with the revalidation of *Acritocentris*, *Exallocentris* and *Xerocentris*, the proposition of a new subgenus for the *Centris hyptidis* group and for the South American clades and rearrangement of other North American species. The revalidation of *Schistemisa* would solve the paraphyly of the currently recognized subgenus *Melacentris*. In *Epicharis*, all subgenera are monophyletic except for *Epicharana*, which has *Epicharis* s.s. nested inside it. Further propositions of new classifications based on the present results would require a deeper study of morphological characters that facilitate the recognition of the newly proposed clades.

Keywords: Hymenoptera, Apoidea, oil bees, *Centris*, *Epicharis*, Neotropical

INTRODUCTION

Comprising about 17500 described species of pollen feeding wasps bees form a well-established clade (Michener 2007). In the last fifteen years, our understanding of bee's phylogeny and evolution has made great progress due to increasing use of nucleotide sequences and model-based phylogenetic methods (Danforth et al. 2013). Molecular data have provided new insights into phylogenetic relationships among families (Danforth et al. 2006a,

2006b); within families (Almeida and Danforth 2009; Cardinal et al. 2010; Litman et al. 2011); and within tribes (Hines 2008; Schaefer and Renner 2008; Rasmussen and Cameron 2010). Although being an active field of research, bee's systematics has been focused on particular groups, especially on social species, and neglected the vast majority of solitary bees (Engel 2011).

The present work focuses on two genera of solitary bees: *Centris* and *Epicharis*, traditionally placed together in one tribe, the Centridini (Michener 2007). They are important components of Neotropical bee fauna due to its number of species, large body size, wide distribution and interaction with many important plant groups (Maués 2002; Sigrist and Sazima 2004; Oliveira and Schlindwein 2009; Gaglianone et al. 2010). *Centris* and *Epicharis* form the most ancient lineage of floral oil-collecting bees (Chapter 1), primarily associated with Neotropical Malpighiaceae, a 90 Ma history of co-divergence (Chapter 3). Studies based on larval morphology (Roig-Alsina and Michener 1993; Straka and Bogusch 2007) and molecular based studies (Cardinal et al. 2010) have indicated the paraphyly of Centridini in relation to the corbciulates. However, these studies focused on the relationships of Apidae family and presented a low sampling of *Centris* and *Epicharis*. To test the monophyly of Centridini as well as the inner relationships of both genera, we sampled 72 species out of 230 of *Centris* and 22 out of 35 of *Epicharis* plus 76 other Apinae (Chapter 1). We found out with high statistical support that *Centris* is sister to the corbiculate bees, and *Epicharis* is sister to both. This findings contradict the cladistic analysis of Roig-Alsina and Michener (1993) that found some synapomorphies for Centridini, most linked to their oil-collecting apparatuses.

Most female *Centris* and all *Epicharis* possesses oil-collecting apparatuses on two or four legs adapted for oil harvesting on flowers (Neff and Simpson 1981). They use floral oils instead of or associated to nectar on the pollen mass for larval food, for nest defense and collection of other nest materials (sand, wood chips) (Vinson et al. 1995). *Centris* has a wide distribution in South America, Central America, and southern North America, occurring in xeric habitats, where many species have lost oil collecting structures and behavior (Neff and Simpson 1981). While all *Epicharis* collect oil exclusively on Malpighiaceae flowers, some oil-collecting *Centris* explore a wide range of Neotropical oil flowers (Vogel 1974; see also Chapter 3).

Centris comprises twelve subgenera: *Aphemisia*, *Centris*, *Hemisiella*, *Heterocentris*, *Melacentris*, *Paracentris*, *Penthemisia*, *Ptilocentris*, *Ptilotopus*, *Trachina*, *Wagenknechtia* and *Xanthemisia; Epicharis* comprises tree monotypic subgenera, *Anepicharis*, *Parepicharis* and *Triepicharis* and six others: *Cyphepicharis*, *Epicharana*, *Epicharis*,

Epicharitides, *Epicharoides*, *Hoplepicharis* (Moure et al. 2012). Three doctoral theses have investigated the phylogenetic relationships among subgenera of *Centris* and *Epicharis*. Gaglianone (2001) tested the phylogenetic relationships among species of *Epicharis* and *Epicharana* and found both subgenera to be monophyletic. Ayala (1998) was the first to study the phylogenetic relationships within Centridini using a *bauplan* concept that precluded the test of validity of each subgenera. However, he tested relationships among subgenera, defining three main groups in *Centris*: 1. *Centris* group, comprising *C*. (*Centris*), *C*. (*Paracentris*), *C*. (*Xanthemisia*) and other subgenera that latter were synonymized in *Paracentris* (Zanella 2002); 2. *Trachina* group, comprising *C*. (*Hemisiella*), *C*. (*Heterocentris*) and *C*. (*Trachina*); and 3. *Melacentris* group, comprising *C*. (*Melacentris*), *C*. (*Ptilocentris*), *C*. (*Wagenknechtia*), *C*. (*Ptilotopus*), *C*. (*Schistemisia*) and *C*. (*Aphemisia*). Vivallo (2010) used a wider sampling in *Centris* and *Epicharis* finding *Melacentris* and *Paracentris* to be paraphyletic. Some of the subgenera of *Centris* and *Epicharis* were not tested, but assumed to be monophyletic *a priori* by the inclusion of only one representative.

In the present Chapter, we present a comprehensive phylogeny for the subfamily Apinae with a dense sampling of *Centris* and *Epicharis* representing all 12 subgenera of *Centris* and all eight of *Epicharis* (Moure et al. 2012), mostly with >2 species, but in the case of subgenera *Ptilocentris* and *Parepicharis* with only 1 species (subgenera *Anepicharis*, *Cyphepicharis* and *Triepicharis* are monospecific). My findings in this Chapter mostly aim at contributing to a new classification of *Centris* and *Epicharis* and their subgenera, and a better undestanding of the evolution of the two groups, now treated as independent lineages in the subfamily Apinae (Chapter 1).

MATERIAL AND METHODS

Taxon sampling and phylogenetic analysis methods are the same as presented in Material & Methods section of Chapter 1.

RESULTS AND DISCUSSION

Phylogenetic relationships within Apinae

Bayesian and Maximum likelihood agreed in general three topology in the phylogeny for the subfamily Apinae rooted in Megachilinae (Fig. 1; for ML phylogeny see Chapter 1,

Fig. A2). Tree major clades can be recognized in Apinae confirming previous studies on the group (Fig. 1): 1. *Cleptoclade* (1 BPP: Bayesian posterior probability; 79% BS: bootstrap support ML tree) (Cardinal et al. 2010; Hedtke et al. 2013): formed by tribes classified as the Nomadinae (Michener 2007) and other cleptoparasites (e.g. Ericrocidini, Rhathymini, *Coelioxoides*) together with Anthophorini; 2. *Xylocopini + Eucerini lines* (1 BPP; 43% BS) (Roig-Alsina and Michener 1993; Silveira 1993; Cardinal et al. 2010; Hedtke et al. 2013): mostly formed by apid tribes formerly classified in the "Anthophoridae", for example Exomalopsini, Tapinotaspidini, Ancylini, Eucerini (Eucerini line) and Xylocopini, Ceratinini (Xylocopini line); 3. *Apine line* (1 BPP; 88% BS) (Silveira 1993; Cardinal et al. 2010; Hedtke et al. 2013) formed by *Epicharis, Centris* and the corbiculate tribes Apini, Meliponini, Bombini and Euglossini. The relationships within the corbiculates found in my results was similar to the most recent hypothesis for this group: Meliponini+Bombini and Euglossini+Apini (Kawakita et al. 2008; Cardinal and Danforth 2011).

For the first time *Ctenoplectrini* and *Tetrapedia* were found to be mutually monophyletic (0.97 BPP; 57% BS) in all analysis but with a controversial position in the phylogeny of Apinae, either as sister to Apine line (Fig. 1) or to the Xylocopini+Eucerini lines clade (Chapter 1; Fig. A2). Although had never been recovered, a mutual monophyly between these two intriguing groups of oil-collecting bees was suggested by Alves-dos-Santos et al. (2002) when observing a few similarities among the nests of the two groups. Both groups have a history of controversial placement in the bee's phylogeny.

Ctenoplectrini was before considered a separated family (Ctenoplectridae) (Alexander and Michener 1995) or as a tribe (Roig-Alsina and Michener 1993). Schaefer and Renner (2008) Ctenoplectrini's molecular phylogeny did not recover a relationship with *Tetrapedia*, probably because of low outgroup sampling, but indicates sister-group relationships between the cleptoparasitic *Ctenoplectrina* and *Ctenoplectra*. *Tetrapedia* is classified in the tribe Tetrapediini, together with *Coelioxoides* (Michener 2007), a cleptoparasitic genus that have been constantly associated to other cleptoparasitic apid bees in recent studies (Straka and Bogusch 2007; Cardinal et al. 2010). Considering our results, Ctenoplectrini and *Tetrapedia* has a long history of divergence, occurred around 86 Mya in the Upper Cretaceous (Chapter 1: Fig. 1 and Fig. A5), which can explain why the morphology has never indicated this association. Nowadays *Tetrapedia* is a Neotropical group associated with oil flowers of Malpighiaceae and Plantaginaceae (Vogel 1974; Martins et al. 2013); while *Ctenoplectra* ocours in Africa and Asia and is oligolectic in Cucurbitaceae flowers from which they obtain oil, pollen and nectar (Vogel 1990).

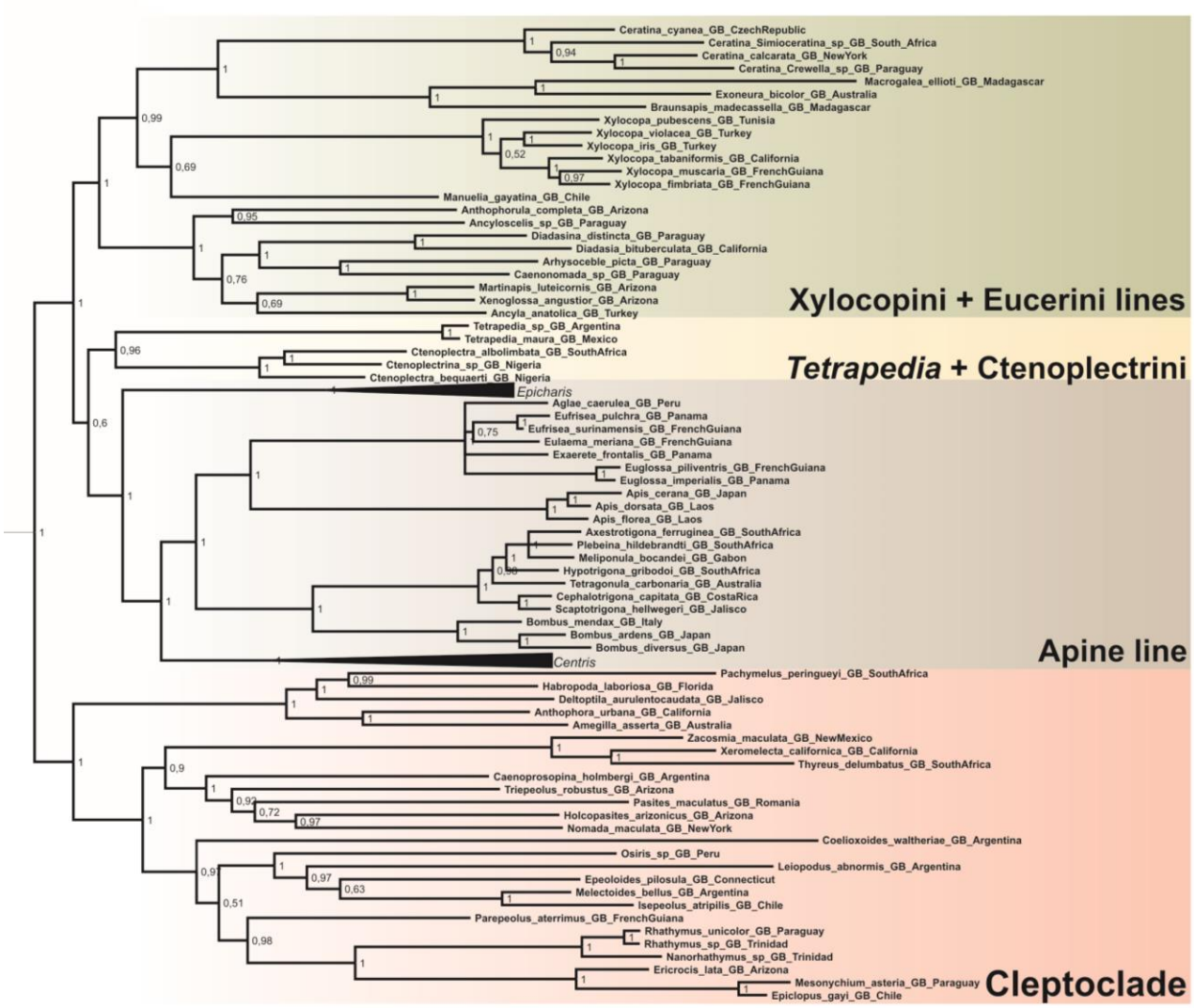

Fig. 1: Bayesian consensus tree resulting from the analysis of the complete data (174 taxa and 4300 aligned nucleotides), rooted on Megachilinae as outgroups. The genera *Epicharis* and *Centris* were colapsed to better shown the other relationships within Apinae. The highlights related to main groupings in Apinae. The figure shows posterior probability values at nodes.

Phylogenetic relationships within *Epicharis*

Epicharis is a well-supported monophyletic group (1 BPP; 100% BS), but the results partially reject the subgenera classification. On the six non-monotypic subgenera proposed for *Epicharis*, only *Epicharana* appear as paraphyletic, being the subgenus *Epicharis* nested inside it (Fig. 2). This position contradicts with other hypothesis that strongly support the monophyly of *Epicharana* and *Epicharis s.s.* based on morphological characters (Gaglianone 2001). Vivallo (2010) in his phylogeny of Centridini, accepted the previously found monophyly of both genera, included only one representative of each, precluding any test of monophyly among *Epicharis* s.s. and *Epicharana*. Gaglianone (2001) included a better

representation of both subgenera than the present analysis. Increasing the sampling in both subgenera would possibly give a different result or simply define the better solution.

The group *Epicharis* formed by *Hoplepicharis*, *Epicharis* and *Epicharana* is well supported (Fig. 2; 1.00 BPP; 100% BS) as indicated by morphological characters (Ayala 1998; Gaglianone 2001; Vivallo 2010). Ayala (1998) found another grouping among all the remaining subgenera that he called as *Epicharoides* group. This grouping is not corroborated in the present analysis neither by Vivallo (2010). Sister to the "*Epicharis*" group, my phylogeny recovered a clade formed by the monotypic *Cyphepicharis* and *Epicharitides* (1.0 BPP; 96% BS) contradicting the *Epicharitides+Epicharoides* clade found before (Vivallo 2010). *Parepicharis* is well supported as sister to *E*. (*Anepicharis*) *dejeanii* (1.0 BPP; 98% BS). The clade *Epicharoides* and *Epicharis* (*Triepicharis*) *analis* form a polytomy with the remaining groups (Fig. 2). Vivallo (2010) did not suggest any change on the classification of *Epicharis.* On the other hand, the present results indicate the need of review of classification of species in *Epicharis* and *Epicharana*.

Epicharis contrasts with the related genus *Centris* by the total dependence of Malpighiaceae flowers and consequently monotony in terms of oil-collecting apparatus and behavior. This association could explain why *Epicharis* has so few species (35) when comparing with *Centris* (230) (Chapter 3). The close association with malpigh flowers certainly also influenced the restriction of *Epicharis* to the humid habitats of Neotropical region. Malpighiaceae occurs extensively in Neotropics, part of Africa and Asia, but always in humid and warm environments (Anderson 1979).

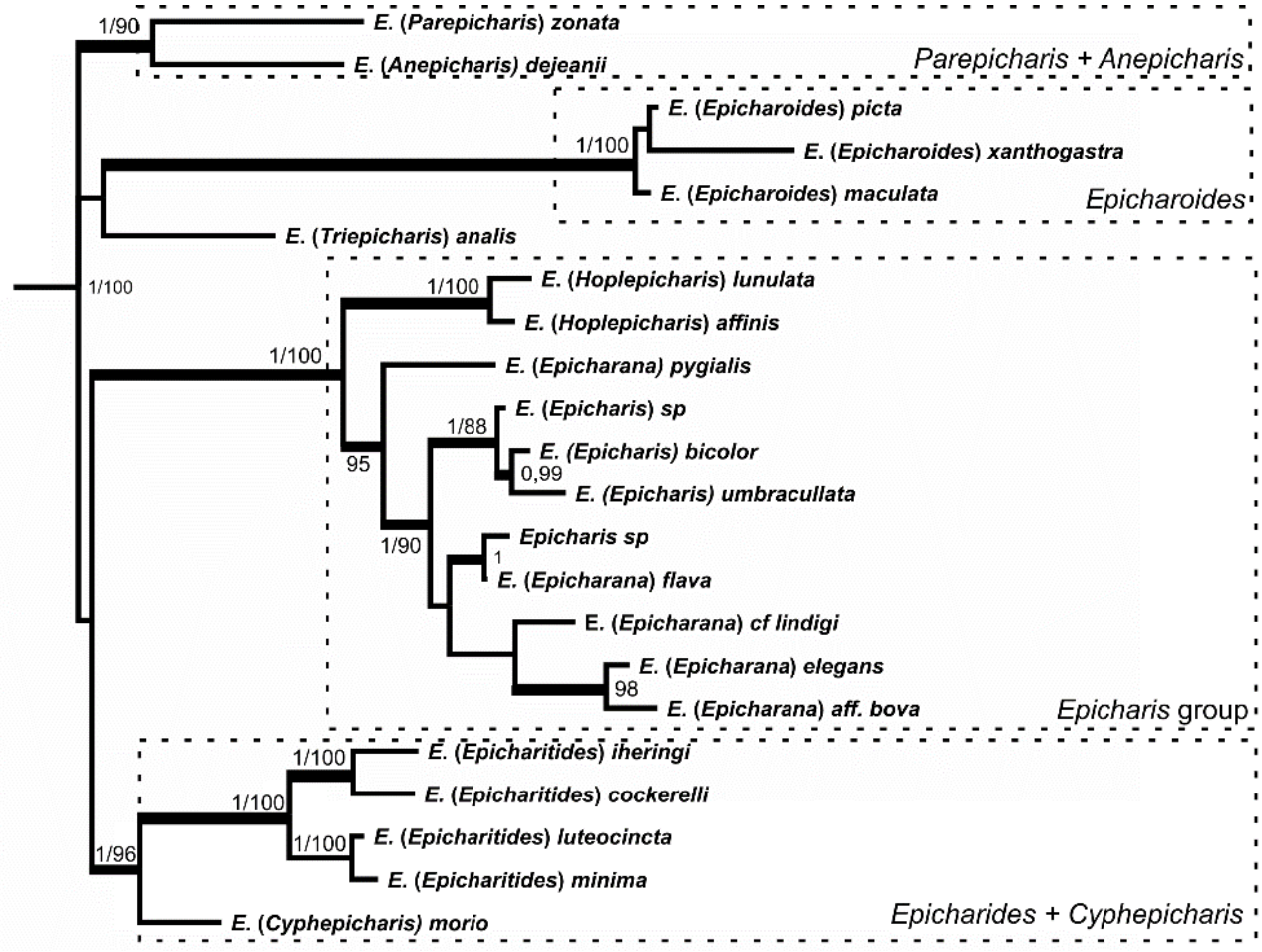

Fig. 2. Detail of the Bayesian consensus tree resulting from the analysis of 174 taxa and 4300 aligned nucleotides for the subfamily Apinae showing the relationships in *Epicharis*. Bayesian Posterior probability values ≥98% and maximum likelihood bootstrap support values ≥70% are shown at nodes. Thickened branches are supported either by ≥98% BPP and/or ≥70% BS. Highlights relate to the accepted subgenera classification (Moure et al. 2012).

Phylogenetic relationships within *Centris*

The results partially rejects the traditional subgenera classification for *Centris*. On the 12 subgenera proposed for *Centris*, seven are monophyletic with high support (1.0 BPP; 100% BS): *Ptilotopus*, *Penthemisia*, *Centris*, *Xanthemisia*, *Hemisiella*, *Heterocentris* and *Wagenknechtia*. *Trachina* appear as monophyletic with low support in the ML tree or as paraphyletic in the Bayesian analysis, apparently by the unstable position of *C.* (*T.*) *aff. ocellaris*. *Paracentris* and *Melacentris* are paraphyletic as currently circumscribed as already indicated by Vivallo (2010).

The first split in the genus *Centris* separate a well-supported clade formed by *C.* (*Hemisiella*), *C.* (*Heterocentris*), *C.* (*Trachina*), *C.* (*Wagenknechtia*) and *Centris* (*Paracentris*) *hyptidis* (*Trachina* group) (1.00 BPP; 85% BS) from the remaining subgenera. We can observe other two main clades, mutually monophyletic in both analysis, however with low support (0.56 BPP; 65% BS). Each of these groups are well supported. First the *Centris* group (sensu Ayala 1998) formed in our analysis by *Paracentris* (in part), *Penthemisia*, *Xanthemisia* and *Centris* (1.00 BPP; 100% BS). Second the *Melacentris* group (sensu Ayala 1998) formed by *Melacentris*, *Aphemisia*, *Ptilotopus* and *Ptilocentris* (1.00 BPP; 100% BS).

Trachina group

The group *Trachina* comprises the subgenera *C.* (*Trachina*), *C.* (*Hemisiella*), *C.* (*Heterocentris*), *C.* (*Wagenknechtia*) and the *C.* (*Paracentris*) species related to *Centris hyptidis* (Fig. 3). This grouping differs from *Trachina* group of Ayala (1998) by the inclusion of *Wagenknechtia* and *Centris hyptidis* group. Vivallo (2010) results differ on the position of *Wagenknechtia* and *hyptidis* but consistently recover the close relationship among *Trachina*, *Heterocentris* and *Hemisiella*.

All subgenera of *Trachina* group have the particular trait of have being freed from the total dependence on Malpighiacae flowers being also adapted to collect oil in Plantaginaceae flowers (Vogel and Machado 1991; Sérsic and Cocucci 1999; Martins et al. 2013). This feature possibly was a key factor in the occupation of driest parts of South America occurred in the *Wagenknechtia* and *hyptidis* group. *Wagenknechtia*, with 7 species occurs in the xeric habitats of Chile and Argentina (Vivallo 2013) and the three species of *Centris hyptidis* group occupy Seasonally Dry Forests, Savannas and Chaco (Vivallo and Melo 2009). These two groups are unique in *Centris* by exploring exclusively other oil sources then malpighs for which they possess two-legged oil-collecting apparatuses contrasting with the four-legged ancestral pattern (Neff and Simpson 1981; see also Chapter 1). In fact, species of *Wagenknechtia* are specially adapted to *Monttea* (Plantaginaceae) and *Calceolaria* (Calceolariaceae) flowers, while *C. hyptidis* are known to mainly visit Angelonieae flowers and *Krameria* (see Chapter 3 and 4). Differing from other *Centris* some species in these group nests on pre-existing cavities (Frankie et al. 1993).

Melacentris group

The group *Melacentris* comprises the subgenera *C.* (*Melacentris*), *C.* (*Aphemisia*), *C.* (*Ptilocentris*) and *C.* (*Ptilotopus*) (Fig. 3). The unique difference between what I present here

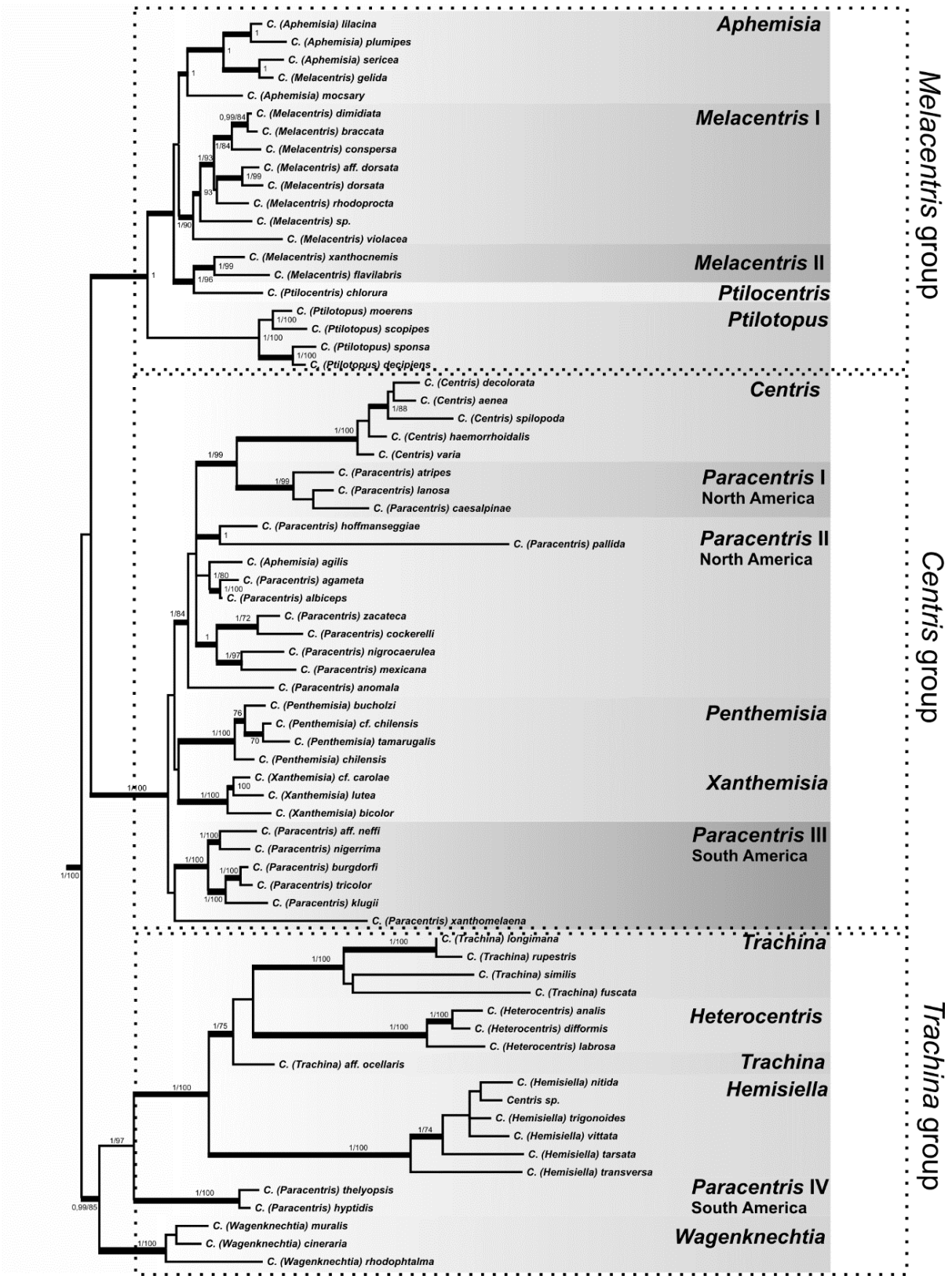

Fig. 3. Detail of the Bayesian consensus tree resulting from the analysis of 174 taxa and 4300 aligned nucleotides for the subfamily Apinae showing the relationships in *Centris*. Subgenera names follow the traditional classification (see Moure et al. 2012). Bayesian Posterior probability values ≥98% and maximum likelihood bootstrap support values ≥70% are shown at nodes. Thickened branches are

supported either by ≥98% BPP and/or ≥70% BS. Highlights relate to well-supported subgenera relationships (groups).

as *Melacentris* group and the homonym group from Ayala (1998) is the exclusion of *Wagenknechtia*. The same configuration for this clade was found by Vivallo (2010). Each component of this group is monophyletic except for *Melacentris* as currently circumscribed. The revalidation of the subgenus *Schistemisia* (Ayala 2002) would solve part of the problem of paraphyly of *Melacentris*, since some of the species previously classified in these subgenus (in my sampling *C. flavilabris*) form a clade sister to *Ptilocentris* (*Melacentris* II). The position of *Centris* (*Melacentris*) *gelida* within *Aphemisia* could be treated as a misclassification of this species in *Melacentris* or paraphyly of both, *Aphemisia* and *Melacentris*. Another case probably due to misclassification is the position of the North-American species *Centris agilis* in *Aphemisia* since in my phylogeny this species is grouped together with North American *Paracentris* (*Paracentris* II, Fig. 3). In my sampling, with revalidation of *Schistemisa*, only the grouping *Melacentris* I (Fig. 3) would be assigned to the subgenus *Melacentris*. Vivallo (2010) suggests the synonymization of *Aphemisia* in *Melacentris*, treating this last as a big subgenus comprising all variants previously classified also in *Schistemisa* by Ayala (2002). Further examination of morphological characters could solve this taxonomical problem.

Melacentris group as far as we known are dependent on oil flowers of Malpighiaceae and holds the basic four-legged apparatuses adapted to these flowers (Chapter 1). In this group we can find some of the largest neotropical bees, especially on *Melacentris* and *Ptilotopus* (Michener 2007).

Centris group

The *Centris* group comprises part of *Paracentris*, *Centris*, *Xanthemisia* and *Penthemisia* (Fig. 3) and totally agrees with *Centris* group from Ayala (1998). In this context the subgenus *C.* (*Centris*), *C.* (*Xanthemisia*) and *C.* (*Penthemisia*) are monophyletic while *Paracentris* are paraphyletic as previously reported (Zanella 2002; Vivallo 2010). *Xanthemisia* and *Penthemisia* appear as sister clades with low support in the Bayesian phylogeny, but in the ML *Penthemisia* is basal to the whole group containing the North-American *Paracentris* and *Centris s.s.* (see Chapter 1, Fig. A2). *Centris s.s.* in both analysis are nested inside the North American *Paracentris* with high support (1.00 BPP; 84% BS). *C. caesalpinae*, *C. lanosa* and *C. atripes* are well supported as sister to the subgenus *Centris* (1.00 BPP; 98% BS).

The North-American *Paracentris* (*Paracentris* II, Fig. 3) would form a clade only with the inclusion of *Centris* s.s. Among the subgenera proposed by Snelling (1974) and further synonymized in *Paracentris* (Zanella 2002) my phylogeny gives support to revalidation of *Xerocentris*, represented by *C. hoffmanseggiae* and *C. pallida* (1 BPP; 63% BS); *Exallocentris* composed only by *C. anomala*, which form a basal lineage of the North American clade (1 BPP; 84% BS); and *Acritocentris* represented by *C. agameta* and *C. albiceps* (1 BPP; 100% BS). However only a careful examination of the morphological characters supporting this tribe would support a robust new classification of these groups.

The South-American species of *Paracentris* grouped together (0.91 BPP; 68% BS), but excluding *Centris xanthomelaena* increase considerably the support (1 BPP; 100% BS). Zanella (2002) found a sister relationship between *C. xanthomelaena* and *Centris* s.s. proposing the need of a new subgenus to accommodate this species, which was never proposed. A careful exam of morphological characters of other *Paracentris* from South America not included in this analysis would help to consider the unity of the South American lineage in a clade and help the classification of these species in a new circumscription of *Paracentris*. Currently classified in *Paracentris* (Moure et al. 2012), but already indicated as a distinct group (Roig-Alsina 2000; Vivallo and Melo 2009), the South American species in the *Centris hyptidis* group in our phylogeny are even less related to the remaining *Paracentris*, belonging to the *Trachina* group (see above).

The *Centris* group is unique among the *Centris s.l.* species by the high number of species that colonized North America: basically all above cited clades of "*Paracentris*", including *Xerocentris*, *Exallocentris* and *Acritocentris* occur exclusively in Neartic Region associated to xeric habitats (Snelling 1974, 1984). Another interestingly trait is the absence of oil-collecting apparatuses, and consequently the non-dependence of floral oils in many of these species, including all representatives of "*Xerocentris*" and *C. ("Exallocentris") anomala*, which represents secondary loss since it is still observable some vestigial structures (Neff and Simpson 1981; see also Chapter 1, Table A.7). Two species classified in *Penthemisia* also have lost the oil-collecting apparatus which motivated Snelling (1974) for classifying the South American *C. mixta* in *Xerocentris* together with other non-oil-collecting North American species. Neff and Simpson (1981) disagreed with Snelling's inference by observing an advanced state of loss of oil collecting setae in *Xerocentris* indicating an older event of loss of oil-collecting behavior. The dissociation between *C. mixta* and *Xerocentris* was further confirmed by Zanella (2002) and by our results (Fig. 3).

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CAPÍTULO 3

SUCCESSIVE RADIATION OF OIL PLANTS IN THE NEOTROPICS AND THE EVOLUTION OF OIL COLLECTING HABITS IN *CENTRIS* AND *EPICHARIS*

SUCCESSIVE RADIATION OF OIL PLANTS IN THE NEOTROPICS AND THE EVOLUTION OF OIL COLLECTING HABITS IN *CENTRIS* AND *EPICHARIS*

ABSTRACT

Angiosperms and insects interact for over 130 million years since the first flowering plants appear offering pollen and/or nectar to these animals. Nowadays many insect-flower interactions are specialized, involving mutual dependencies and adaptations between partners. The floral oil syndrome evolved in a mix of specialized and generalized interactions in a highly dissimilar number of oil-collecting bees and oil offering plants. Malpighiaceae is the oldest and most species rich family to produce floral oil in the Neotropics and pollinated mainly by females of *Centris* and *Epicharis.* Considering the availability of a robust phylogeny and dating for these bee genera (Chapter 1), I estimated divergence times for the other oil producing families in the Neotropical Region to reconstruct the framework of interactions among bees and plants though time. Malpighiaceae divergence times were estimated in previous work; for Plantaginaceae, I performed sequencing, phylogenetics and dating (Chapter 4). For Calceolariaceae, Krameriaceae, Iridaceae and *Nierembergia* I downloaded sequences already published and estimated divergence times using substitution rates for plastid and nuclear markers. I found out that the oldest oil-producing plant clades, Malpighiaceae, Plantaginaceae and Krameriaceae are primarily associated with the oldest groups of oil-collecting bees, *Centris* and *Epicharis*. Other oil sources, here dated as originated in the Miocene, Iridaceae and *Nierembergia*, are sporadic partners of *Centris*, but mainly pollinated by a younger group of oil bees, Tapinotaspidini. While the *Epicharis* lineage stuck on the exploitation of Malpighiaceae flowers, *Centris* occupied new habitats and associated with other plants, developing different morphologies to exploit new oil sources. In driest parts of Neotropical and Nearctic Region, in different time periods, *Centris* freed from the dependence on oil flowers, and stopped the oil collecting, certainly saving much energy. This event was probably facilitated by the dry condition of the soil for nesting, which does not require the waterproofing characteristics of the floral oils, as in humid tropics.

Keywords: oil bees, Neotropical, Malpighiaceae, Plantaginaceae, molecular clock

INTRODUCTION

The history of flowers and insect interactions is particularly old: about 130 million years ago (Brenner 1996) or even more (Doyle 1969; Hochuli and Feist-Burkhardt 2004, 2013; Zavada 2007), the first flowering plants appear immediately interacting with nectar/pollen feeding insects. The first animal pollinators were generalist, probably by the non-exclusionary morphology of the first flowers (Grimaldi 1999). The present array of specialization among angiosperms and insects, particularly bees, developed through an mix of synchronous coevolution and opportunism in exploit the flower sources and pollinator preferences already present (Armbruster 1997; Ramírez et al. 2011; Schiestl and Dötterl 2012). Nowadays most angiosperms are pollinated by animals, insects in particular, where bees are the most important agent (Ollerton et al. 2011).

Specialization defines many angiosperm-insect interactions: a combination of floral traits and pollinators preferences and/or abilities, which can culminate in extreme mutual dependence (Nilsson 1998). On the other hand many interactions are generalists and those play an important role in plant-pollinator communities (Waser et al. 1996). Phylogenetic evidence shows many cases of generalized systems originated from specialization (Sipes and Tepedino 2005; Michez et al. 2008), but the contrary can also occur (Tripp and Manos 2008). Although specialization can lead to more efficient pollination and use of resources, it is also a risky condition. Therefore, coevolution is not only adaptation to each other, but a case of scape from total dependence in one source (Ehrlich and Raven 1964).

The floral oil syndrome involves a mix of specialized and generalized interactions in the context of coevolution with a highly dissimilar number of plant and bee species involved (Vogel 1974; Renner and Schaefer 2010). About 1600 species of plants produce floral oils as a reward to their pollinators, belonging to 11 families: Malpighiaceae (1300 species) in which oil is the ancestral reward system, the remainder to the Orchidaceae, which have oil-offering species in many genera, Calceolariaceae (~200, all in *Calceolaria*), Cucurbitaceae (~102 in several genera), Myrsinaceae (~75, all in *Lysimachia*), and just over a dozen small genera of Iridaceae, Krameriaceae, Stilbaceae, Scrophulariaceae, Solanaceae, and Plantaginaceae (Vogel 1974, 1988; Rasmussen and Olesen 2000; Machado 2004; Renner and Schaefer 2010). Moreover about 450 species of bees collect floral oils and act as pollinators of those plants.

Most of oil producing plants occurs in Neotropics (see Fig. 1), mainly by the great predomination of oil Malpighiaceae in that region, and involves four groups of bees: *Centris* and *Epicharis* (traditionally “Centridini” tribe, Chapter 1 and 2), Tapinotaspidini and

Tetrapedia. The mutualism between these bees are often obligatory at family, or less commonly, at genus level, not at species level (Rasmussen and Olesen 2000; Sigrist and Sazima 2004; Martins et al. 2013), in spite of the lack of field observation on bee oil foraging's range. Molecular clock implied Malpighiaceae started about 75 Mya the production of oil, ancestral in this family (Renner and Schaefer 2010; Xi et al. 2012), and the interaction with apid bees. Successively other plant families entered this system (Renner and Schaefer 2010), but when and how is vaguely understood by the lack of robust phylogenies of the partners. Partially answered by Renner and Schaefer (2010), this build up could only be completely understood with a complete and robust phylogeny for the main pollinators of Neotropical oil flowers, i.e. *Centris* and *Epicharis,* now available (Chapter 1 and 2), allowing the construction of this framework of association.

Certainly the oil-bee-flower history started with Malpighiaceae, but how the build-up of oil plants and bees interactions in the neotropics occurred? At which time did *Centris* start to interact with other oil families?

MATERIAL & METHODS

Phylogenetic information and molecular dataset for bees and plants

For dating and ancestral state reconstruction purposes I used my own dataset on bees and published molecular datasets for Neotropical oil-producing plants, known to be visited, and sometimes pollinated by *Centris* and *Epicharis* (Fig. 2) (Vogel 1974; Machado 2004; Renner and Schaefer 2010). For Malpighiaceae I used the ages already published in the literature and for the Angelonieae (Plantaginaceae) my own data set (Chapter 4). For other oil hosts of *Centris*, I estimated the divergence times based in already published dataset, since these groups have never been focus of specific dating studies, but only indirect dating from broader sampling studies. I follow describe the groups and main phylogenetic and dating works that have been done on them.

Centris and *Epicharis* (Apidae)

I used phylogeny and the fossil calibrated tree for *Centris* and *Epicharis* performed by Martins, Melo and Renner (Chapter 1), based on a matrix of 174 taxa and 4300 characters from four nuclear markers. For detailed information about dataset and methods, please see Chapter 1.

Fig 1. *Centris* and *Epicharis* interacting collecting oil on flowers. A. *Epicharis cockerelli* on oil-offering *Byrsonima* (Malpighiaceae); B. *Centris tarsata* on oil-offering *Krameria* (Krameriaceae); C. *Centris* (*Hemisiella*) *trigonoides* on oil.offering *Angelonia integerrima* (Plantaginaceae); D. *Centris* (*Centris*) *aenea* on oil-offering *Byrsonima* (Malpighiaceae).

Malpighiaceae

Estimated as the oldest oil producing plants in the world (Renner and Schaefer 2010), Malpighiaceae is a large family comprising about 1300 species, mainly occurring in Neotropics, were it possibly originated further dispersing to Old Word (Davis et al. 2002; Davis and Anderson 2010; Xi et al. 2012). The floral oil production is basal in Malpighiaceae and it was lost in African and Asian species (Davis and Anderson 2010), but most Neotropical species continues to reward pollinators with oil produced in four pairs of glands in the corolla

(Vogel 1974; Sigrist and Sazima 2004). The origin of Malpighiaceae has been estimated in about 65 My (Davis et al. 2002), but more recent estimates for all Malpighiales indicated a age for the split between Malpighiaceae and Elatinaceae at 86 My (100-73) and Malpighiaceae's crown age of 60 (69-52) (Xi et al. 2012).

Angelonieae (Plantaginaceae)

The tribe Angelonieae comprises four oil-producing genera, *Angelonia*, *Basistemon*, *Monopera* and *Monttea*, plus the oil-less *Ourisia* and *Melosperma* (Albach et al. 2005). None of the genera in this group has been object of phylogenetic analysis, except for *Ourisia* (Meudt and Simpson 2006, 2007) until our first assessment on the phylogenetic relationships among them (Chapter 4). Previously classified in the Scrophulariaceae, this group is the only clade in Plantaginaceae that reward pollinators with oil (Renner and Schaefer 2010). The age of the split between *Angelonia* and *Monttea* was estimated as 13 Ma (34-7) (Renner & Schaefer 2010). However our estimates indicate a much older ages for the Angelonieae: 35 (26-47) Ma and 25 (18-34) Ma for the split between *Angelonia* and *Basistemon* (Chapter 4). Phylogeny indicates five independent origins of oil production in Angelonieae: once in the common ancestor of *Angelonia*, once in the common ancestor of *Monttea* and three times in *Basistemon* (Chapter 4). *Centris* bees are the main pollinators of *Angelonia, Monttea* and *Basistemon silvaticus* (Vogel and Machado 1991; Sérsic and Cocucci 1999; Martins et al. 2013). Here each of these origins will be treated separately.

***Nierembergia* (Solanaceae)**

Nierembergia is a genus of Neotropical oil plants, which includes 21 species, unusual by possessing floral oil syndrome of pollination, unique in Solanaceae (Tate et al. 2009). Using molecular (ITS, rps16), morphological and cytogenetic evidences, Tate et al., (2009) found the genus as monophyletic. The split between *Nierembergia* and *Petunia* has been inferred as 12 (38-10) Ma (Renner and Schaefer 2010), while the crown group of Solanaceae has originated 37-38 (26-49) Ma ago (Bell et al. 2010). To infer divergence times in *Nierembergia* we used the molecular matrix of Tate et al. (2009), available in TreeBase (S1780; TreeBase 2014). The matrix is composed by 1639 aligned nucleotides (642 pb ITS and 997 rps16) for 35 taxa. The outgroups included *Petunia* and the sister group of *Nierembergia*, *Bouchetia*. The root was constrained in BEAST analysis to fit with the phylogenetic hypothesis of relationships in the genus**.** Voucher information, including accession numbers and geographic locality, are available in the original work (Tate et al.

2009). Varieties of the same species were excluded from the dating analysis to avoid zero-length branches.

Fig. 2. Floral oil producing plants occurring in Neotropical Region. A. *Peixotoa* sp. (Malpighiaceae), B. *Krameria tomentosa* (Krameriaceae), C. *Angelonia goyazensis* (Plantaginaceae), D. *Nierembergia* sp. (Solanaceae), E. *Cypella herbertii* (Iridaceae), F. *Calceolaria* sp. (Calceolariaceae). Photos: A, B, D, E: Antonio Aguiar.

***Krameria* (Krameriaceae)**

Krameria is a genus of New world plants, single in the family Krameriaceae, with 18 species occurring in arid and semi-arid regions, ten of them in North and Central America and six ranging from Northern Colombia to east-central Brazil (Simpson 1989a). Using morphological and molecular data (ITS, 5.8S), Simpson et al. (2004) reconstructed the phylogeny of the whole family using Zygophyllaceae as outgroup. Previous age estimates for *Krameria* varies considerably, from ca. 12 Ma (Renner and Schaefer 2010) to ca. 62 Ma (Naumann et al. 2013), but none of these studies were particularly focused on Krameriaceae. I used the molecular sequences from Simpson et al. (2004), downloaded from GenBank (AY260972-AY261121) to infer the divergence times in the genus. The final aligned matrix is composed by 800 nucleotides for 20 species, 17 out of 18 species of *Krameria*, plus the outgroups *Guaiacum angustifolium*, *Kallstroemia parviflora* and *Tribulus terrestris* (Zygophyllaceae). Only one sequence per species were maintained.

Iridaceae, with focus on Sisyrinchieae, Trimeziae and Tigridae

Iridaceae is a large family worldwide distributed, with about 2000 species (Goldblatt et al. 2008). Only the Neotropical tribes Sisyrinchieae, Trimeziae and Tigridieae, closely related to each other (Goldblatt et al. 2008), are known to produce floral oil as reward to pollinators (Renner and Schaefer 2010). The estimated age for Iridaceae is 31 -34 Ma (17-49) (Bell et al. 2010) and they possible originated in Australasia with further diversification events, mainly in South Africa and South America (Goldblatt et al. 2008). The divergence times of the Neotropical oil tribes has been inferred as less than 35 Ma (Renner and Schaefer 2010). The Neotropical oil tribes were focus of two other papers (Chauveau et al. 2012; Lovo et al. 2012). Oil production was gained many times in the history of these plants (Chauveau et al. 2012). I performed dating analysis in oil producing Iridaceae using the sequences from Chauveau et al. (2012), which partially overlap with Goldblatt's sequences, representing 97 species in the tribes Sisyrinchieae, Trimeziae and Tigridieae for a total of about 6000 aligned nucleotides for three coding regions (rps4, matK and rbcl), the trnL-F region and rps16 intron (the same used by Goldblatt et al., 2008). The oil glands evolved many times in the Iridoideae, at least two in *Sysirinchium*, one in Trimezieae and seven in Tigrideae. Here we will consider only three of these origins, because they have recorded interactions with *Centris*: *Cipura*, *Cypella* and *Sysirinchium* (Giannini et al. (2013); A. Aguiar, pers. com.); Tapinotaspidini oil bees are the main pollinators of Neotropical oil Iridaceae (Aguiar pers. com.; Cocucci and Vogel 2001; Silvério et al. 2012).

***Calceolaria* (Calceolariaceae)**

Calceolaria, together with *Jovellana* and *Porodittia* are members of the new family Calceolariaceae, previously classified in Scrophulariaceae (Albach et al. 2005; Oxelman et al. 2005). About 200 species are recognized in *Calceolaria*, mostly occurring in the Andean Region, South America, and Central America (Molau 1988). Recent studies reconstructed the phylogenetic relationships in *Calceolaria* using molecular markers, ITS and matk, first with 46 species (Andersson 2006) and further with 103 species (Cosacov et al. 2009). Nylinder et al. (2012) dated the split between *Calceolaria* and *Jovellana*, but got very different ages between plastidial rates: rbcL 19.6 (95% HPD: 6.5 – 40.2); matk 30.8 (14.8–51.3), atpB-rbcl 12.9 (5.1–24.8). Renner and Schaefer (2010) found the crown age of *Calceolaria* to be ca. 5 (6-1) Ma. I used the same DNA sequences of Cosacov et al. (2009), which is the larger phylogeny available for *Calceolaria* so far. In total the matrix had 103 species, plus four outgroups, for a dataset of two markers, matk and ITS. The sequences were downloaded from GenBank.

Estimation of phylogeny and divergence times

Prior to dating analysis each matrix were submitted to phylogenetic analysis. The matrix for *Nierembergia*, downloaded from TreeBase, was maintained with the original alignment (Tate et al. 2009). The remaining dataset, downloaded from Genbank were submitted to alignment in MAFFT (Katoh and Standley 2013), using default parameters: 200PAM/k=2 scoring matrix for nucleotide sequences; gap opening penalty = 1.53; offset value = 0. The sequences were aligned under the automatic option for algorithm, which let the program to decide which one is the best for that dataset depending on the size. Maximum likelihood tree searches and bootstrapping of the combined data using 1000 replicates were performed in RAxML (Stamatakis 2006) using the graphical interface raxmlGUI (Silvestro and Michalak 2012). The trees were visualized, edited and rooted in Figtree (Rambaut 2014). The topology were compared with the previous phylogenetic assessments and, in general, all phylogenies were similar to the previously published (Supplementary material: Fig. S1-S3)

Divergence times were estimated using the Bayesian approach implemented in BEAST 1.7 (Drummond et al. 2012) using a Yule tree prior, the GTR + G substitution model, and the uncorrelated lognormal relaxed clock. I use a relaxed molecular clock as recommended in the manual when ucld.stdev values in Tracer are ≥0.5. The MCMC runs in the Krameriaceae and

Nierembergia datasets were run for 10 million generations. For Iridaceae we run two separated runs of 20 million generations each. For *Calceolaria* matrices we run four different runs ranging from 20 to 80 million generations each, totalizing 210 million generations. All runs were sampled every 1000th generations. Separated runs for Iridaceae and *Calceolaria* were combined in LogCombiner (BEAST package). Convergence of the chains was checked in Tracer. TreeAnnotator (BEAST package) was used to create a maximum likelihood credibility tree.

As there is no fossils for the clades analyzed we use substitution rates for plastid and ITS to calibrate our clock. The plastid rate used was 0.0007 substitution/site/Ma, calculated over entire plastome (Palmer 1991) and for ITS was 0.00427 s/s/Ma, a medium rate calculated from *Plantago* (Plantaginaceae) (Kay et al. 2006). The prior on each rate was a gamma distribution with an initial value of 1.0, and rates were unlinked among the nuclear and plastid partitions.

RESULTS AND DISCUSSION

Age estimates

The age estimates obtained in the present work is presented in Table 1 and chronograms can be seen from Fig. 3 to 5. While Malpighiaceae originated in the Cretaceous (Xi et al. 2012), starting the mutualism with oil bees, the other oil plants began to originate only in the Eocene with Krameriaceae and Plantaginaceae and others in the Miocene (Fig. 6). Krameriaceae's stem presented the older age in our estimates: 50 My (40-59) for the split between *Krameria* and Zygophyllaceae (Fig. 3B). Previous estimates for the age of Krameriaceae varies considerably precluding the cross validation with our result. The previous estimates for the stem age of Krameriaceae were 89-55 Ma (Wang et al. 2009), 62 (29-93) (Naumann et al. 2013) and 70 (49-88) (Bell et al. 2010). For *Krameria's* crown we got 23 (19-28), while in Renner and Schaefer (2010), with a less dense sample, the age for this clade was 12 (5-34). The production of floral oil is ancestral condition in Krameriaceae (Simpson et al. 1977), therefore the second to enter the evolutionary scenario of Neotropical oil flowers.

Table 1. Origins of oil producing flowers in the Neotropics: estimated ages for each group of clades that interact with *Centris* and *Epicharis*. All ages are derived from this study except for Malpighiaceae and Plantaginaceae. (see Material and Methods for references).

Clade	Mean age (95% Highest posterior density - HPD)	
	Crown group	**Stem Group**
Malpighiaceae	60 (59-82)	85 (73-100)
Krameriaceae	23 (19-28)	50 (40-59)
Calceolaria (Calceolariaceae)	8 (6-10)	13 (9-18)
Nierembergia (Solanaceae)	8 (6-11)	20 (14-26)
Plantaginaceae I: *Angelonia*	17 (12-23)	25 (17-34)
Plantaginaceae II: *Monttea*	3,5 (1 - 8)	17 (8-28)
Iridaceae I: *Sysirinchium*	13 (10 – 17)	9 (6 – 11)
Iridaceae I: *Cypella*	1,4 (1 – 3)	7 (4 – 10)
Iridaceae I: *Cipura*	2 (1 – 4)	7 (4 – 10)

For Calceolariaceae we obtained a crown age, i.e. the split between *Calceolaria* and *Jovellana*, of 13 (9-18) Ma (Fig 4). *Calceolaria's* crown age estimates was 8 (6-10) Ma. Variable estimates of Calceolariaceae median age were obtained using different markers ranging from 13 to 31 Ma (Nylinder et al. 2012). Other estimates for the family crown was 15 (27-4) Ma (Renner and Schaefer 2010). The production of floral oil is also ancestral for *Calceolaria*, with some further losses.

We got 22 (17 – 29) Ma for the split between *Petunia* and *Nierembergia* plus sister groups (Fig 3A). Our age fits with previous estimates: Solanaceae crown, 35 My (Dillon et al. 2009) or 38 (26-49) (Bell et al. 2010) and the estimated age for *Petunia* x other Solanaceae: 28 Ma (19-39) (Bell et al. 2010). The oil producing Iridaceae clade, i.e. the tribes Tigridieae, Trimezieae and Sysirinchieae, was estimated to be 28 (22-34) Ma (Fig 5). This age fits with the crown group age for Iridaceae: 34 (20-49) Ma (Bell et al. 2010).

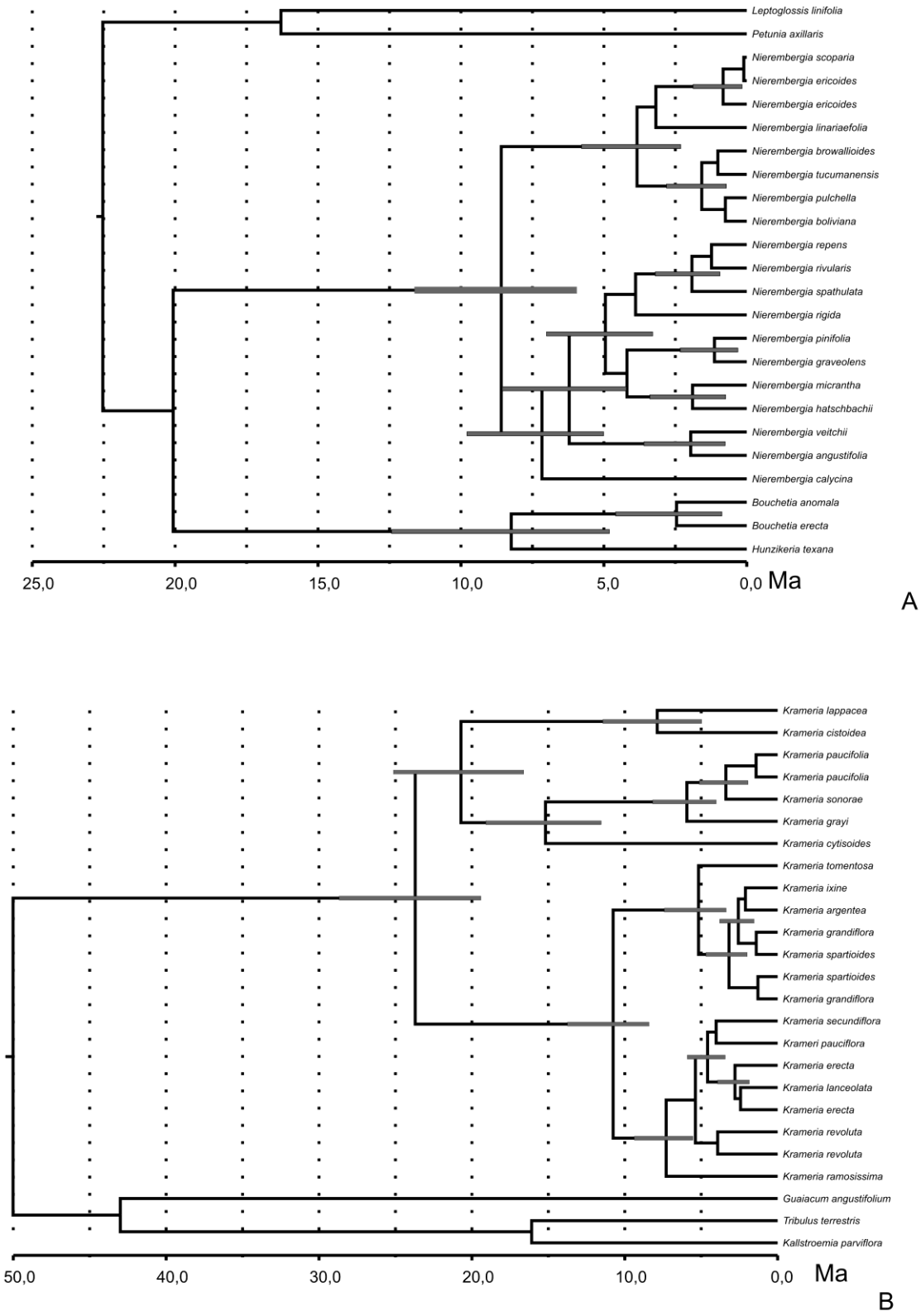

Fig. 3. Chronograms for *Nierembergia* and *Krameria*. **A**. Chronogram for *Nierembergia* rooted in *Petunia* (Solanaceae) obtained under a Bayesian relaxed clock model for a matrix of 35 taxa and 1639 nuclear and plastid aligned nucleotides. **B**. Chronogram for Krameriaceae rooted in Zygophyllaceae obtained under a Bayesian relaxed clock model for a matrix of 20 taxa and 800 nuclear aligned nucleotides. Bars indicate 95% HPD.

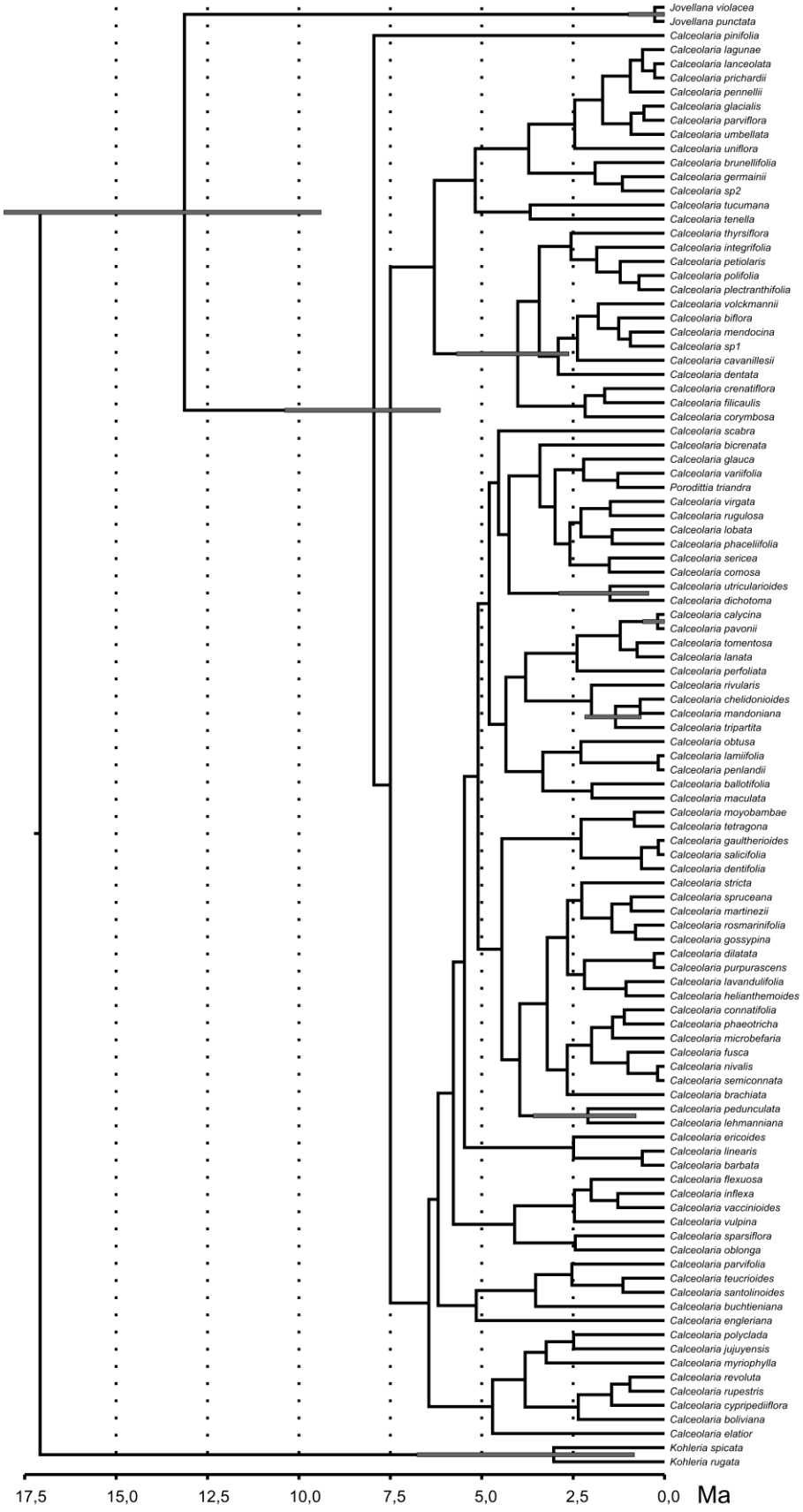

Fig. 4. Chronogram for Calceolariaceae rooted in *Jovellana* obtained under a Bayesian relaxed clock model for a matrix of 104 taxa and 1544 nuclear and plastid aligned nucleotides. Bars indicate 95% HPD.

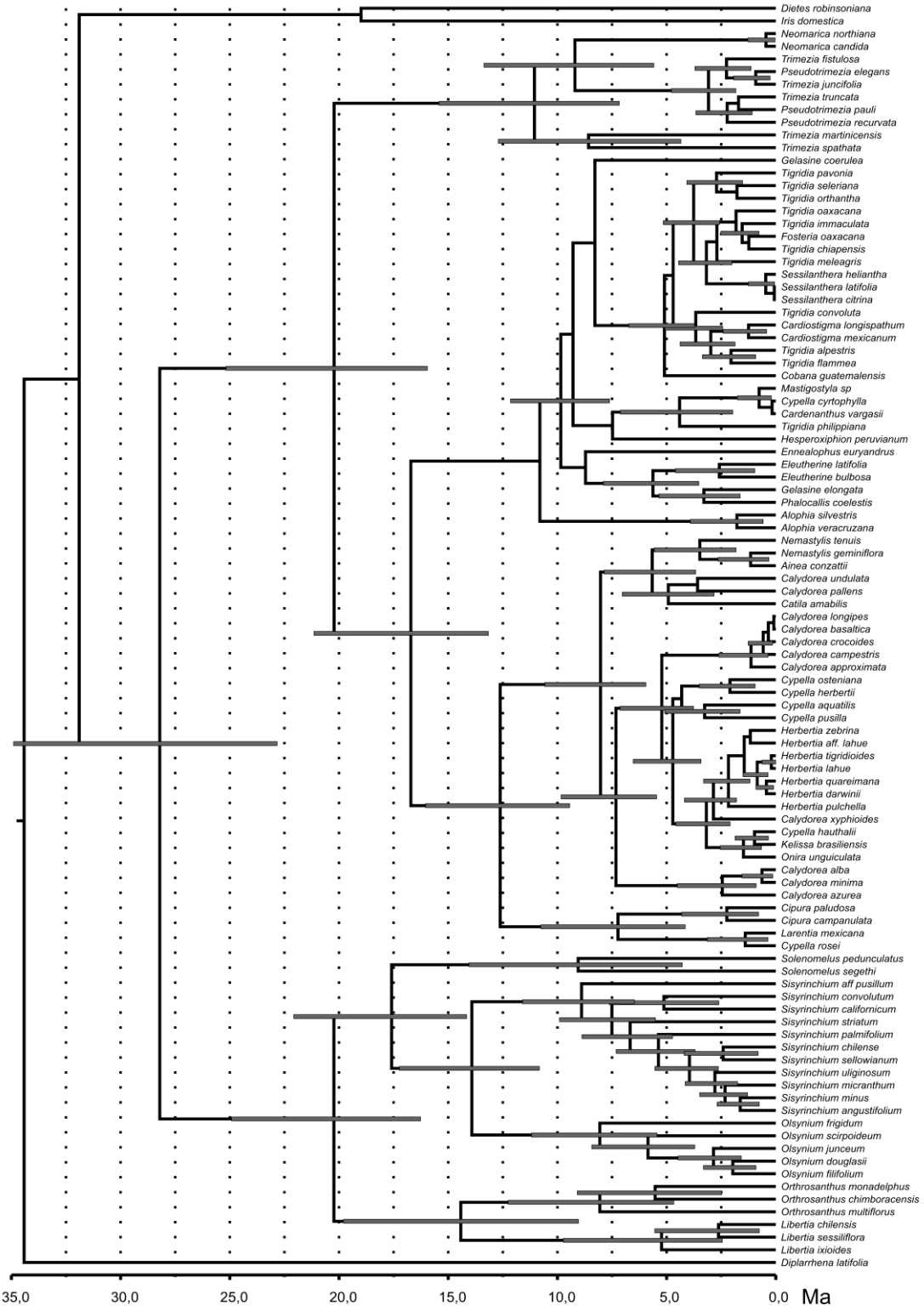

Fig. 5. Chronogram for the Neotropical oil tribes in Iridoideae (Iridaceae) obtained under a Bayesian relaxed clock model for a matrix of 100 taxa and 5905 nuclear and plastid aligned nucleotides. Bars indicate 95% HPD.

Malpighiaceae and the conservatism in *Epicharis*

Malpighiaceae has always being inferred as the first angiosperm family to mutualistic interact with pollinators offering oil as a reward Vogel (1974), further confirmed by age estimates (Renner and Schaefer 2010; Xi et al. 2012). Now, with my molecular clock estimates, we can infer that a bee like *Epicharis* was the first to interact with the first malpigh oil flowers, and maintained this mutualism until the present (Fig. 6). Malpighiaceae's first oil flowers appear at least 60 Ma ago (Malpighiaceae's crown age) or even older, up to 100 Ma, if we consider that this character was present in the ancestral (Malpighiaceae's stem age: Xi et al. (2012). The range of age estimates for malpighs (Table 1), therefore agrees with the estimated age for the Apine line, where the ancestor was an oil-collecting bee (Chapter 1). The apine line ancestor beard a four legged oil apparatus similarly to what we found in the extant *Epicharis* and most *Centris*. Fossil record evidences the antiquity of the malpigh flower morphology and the established relationships with apid bees. The glandular sepals, exclusively present in Malpighiaceae, was already present in *Eoglandulosa warmanensis,* a fossil flower from the Eocene (Taylor and Crepet 1987).

The evolution of floral morphology in neotropical Malpighiaceae was constrained by the closest association with *Centris* and *Epicharis* bees (Davis et al. 2014). They are very conservative in the aspects regarding attracting, orienting, and rewarding pollinators in contrast with other characters, like fruits and habit (Anderson 1979). The hypothesis of floral conservatism in Neotropical Malpighiaceae flowers constrained by pollinator selection was further confirmed by the observation of floral morphology in New and Old world sister clades, where none of the Old word flowers has maintained the constant floral morphology after being freed by the pollinators selection (Davis and Anderson 2010). This constant floral morphology was proven to be due to extrinsic factor (i.e. oil bee pollinators) and not by intrinsic factors (development, genetics), which evidences the tight mutualism between Malpighs and "centridine" bees (Davis et al. 2014).

The four-legged pattern present in *Epicharis* was also probably constrained by the dependence on Malpighiaceae flowers, contrasted with several lineages in *Centris,* which developed other types of oil-collecting apparatuses and started to exploit the younger oil flowers. On the other hand, *Epicharis* females can eventually collect oils from other sources, such as Orchidaceae and Krameriaceae (Neff and Simpson 1981), but how often and how efficient is this behavior is so far unknown. But why *Epicharis* stuck in the oil flowers of

Malpighiaceae while *Centris* evolved several other oil-flower association? A topic for further investigation.

How *Centris* bees entered new mutualism or scape from dependence on oil flowers

The ability to exploit other oil sources is probably correlated to the high diversity of *Centris* (230 spp) comparing to *Epicharis* (35 spp) and to the wide distribution of the first. While *Epicharis* is restricted to the humid areas of Neotropics, such as this main partner Malpighiaceae species, *Centris* can occupy xeric habitats in South America and North America. *Centris* species changed first from the exclusive exploitation of Malpigh flowers to other floral oil sources and latter to the independence of floral oil. Both changes occurred more frequently in driest parts of South America, for example, the deserts of United States and Mexico and the xeric habits of Argentina and Chile (Neff and Simpson 1981). Coincidently or not, these are the habits were Malpighiaceae are scarcer.

The first losses of oil-collecting apparatus and behavior occurred in North American lineages, around 14 Ma ago in *Centris* (*Paracentris*) *anomala* lineage and in the ancestor of *Centris* (*Paracentris*) *pallida* and *Centris* (*Paracentris*) *hoffmanseggiae* (Fig. 6). Younger instances of losses occurred in South America, in *Centris* (*Penthemisia*) *muralis* at ca. 5 Ma ago and in *Centris* (*Penthemisia*) *tamarugalis* at ca. 2 Ma ago (Fig. 6). Neff and Simpson (1981) observed many characters of an oil-collecting ancestor in this South American *Centris* in comparison with the more specialized North American species, indicating the younger instance of loss confirmed by our molecular clock (Chapter 1).

Plantaginaceae oil flowers, which are the second most important oil source for *Centris*, originated and diversified in the arid and semi-arid habitats of South America, with a few species occurring in humid habitats (Chapter 4). Between 15 to 20 Ma ago, in the Eocene, the first oil flowers in Plantaginaceae appear, in *Angelonia* and *Monttea* (Chapter 4). But only in the Miocene, the clades specialized in the Plantaginaceae oil flowers, for example the subgenus *C.* (*Penthemisia*), *C.* (*Wagenknechtia*) and the South American *Paracentris* diversified (Fig. 6). But what signify being specialized in these other oil sources? First, the exclusive records on these and other non-malpigh flowers (Table S1) indicate specialization; second and most important, the loss of combs of hairs and the origin of new ones that, instead of scratch the epithelial glands of Malpighiaceae, would soak the oil from glandular hairs, the

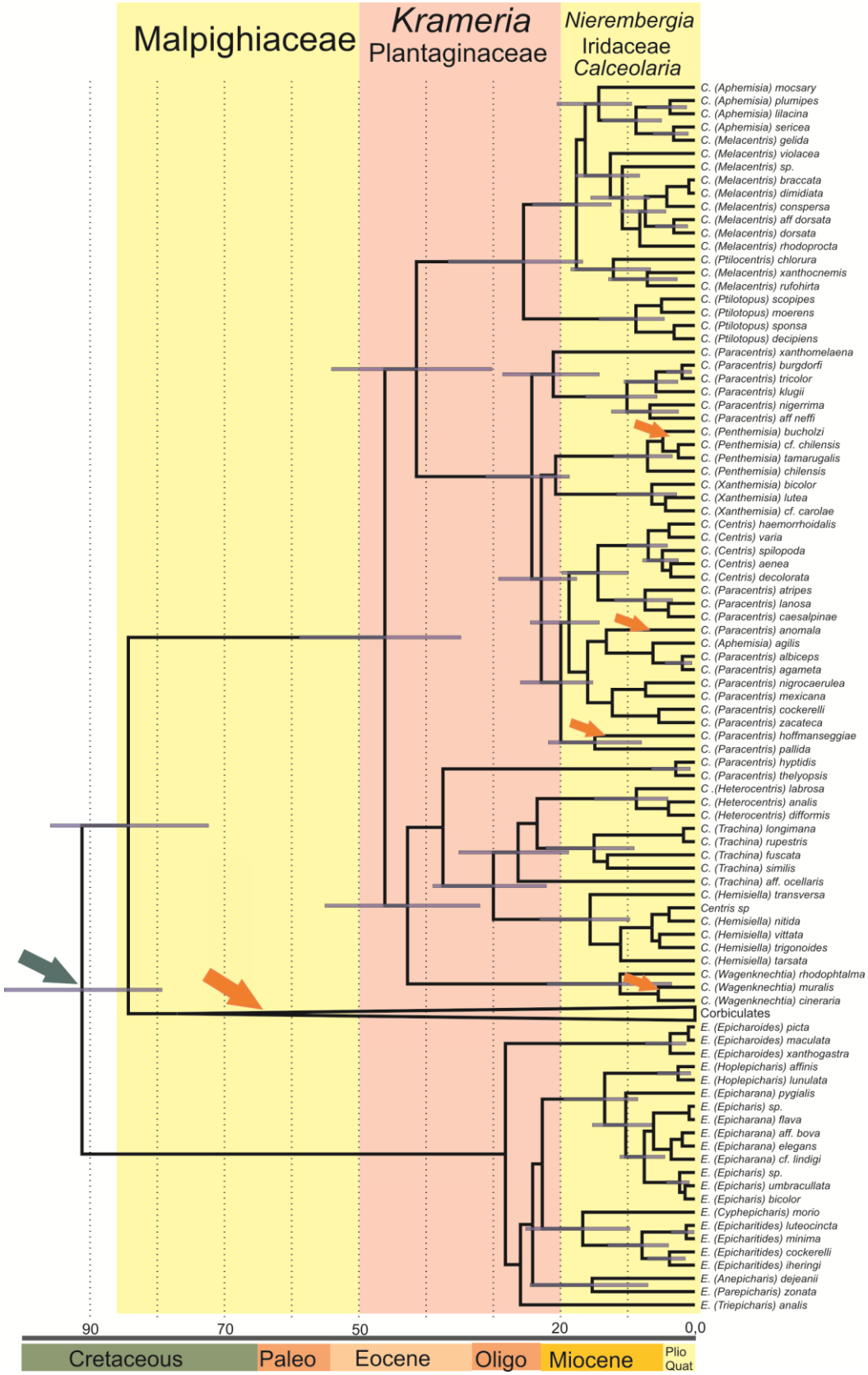

Fig. 6. Part of the time-calibrated phylogeny for corbiculates, *Centris* and *Epicharis*, other Apinae, and Megachilinae (Chapter 1), focused on the apine line. Highlights indicate the time of origin of the main oil sources of *Centris* and/or *Epicharis*. Green arrow indicate the origin of oil collecting in the ancestor of the apine line, orange arrow indicated the loss of this behavior.

kind of glands present in Plantaginaceae and Iridaceae (Vogel 1974). In general, this modification is accompanied by the loss of the second pair of oil-collecting apparatus, only functional in the exploitation of malpigh flowers (Vogel 1974; Neff and Simpson 1981). The dry climate in these regions possibly allowed the survival without oil, since this source is used mainly by waterproofing nest cells in the soil of humid environments (Vinson et al. 2006).

A remarkable case of adaptation to non-malpigh flowers occurred in *Centris hyptidis* and related species, *C. hyptidoides* and *C. thelyopsis*, all of them are pollinators of Plantaginaceae flowers (Machado et al. 2002; Martins et al. 2013), and originated in the Quaternary (Fig. 6). Extremely long front legs adapted to deep corolla tube in *Angelonia* flowers were observed in *C. hyptidis* (Machado et al. 2002). Although this particular long legs were not observed in the other two related species, they also were recorded only on Plantaginaceae flowers and have modified oil-collecting apparatuses (Martins et al. 2013). This group of species diversified in the Dry Diagonal of South America, with extant members occupying the Caatinga of Northeastern Brazil (*C. hyptidis*), Caatinga enclaves of Central Brazil (*C. thelyopsis*) and Chaco of Brazil, Paraguay and Argentina (*C. hyptidoides*) (Vivallo and Melo 2009). They diversified in the same region as the main oil source Angelonieae and synchronously in Pliocene/Quaternary (Chapter 4).

An important oil source for the Andean species are the species rich genus *Calceolaria*, which has more than 200 oil-producing species (Molau 1988; Renner and Schaefer 2010), and diversified in the last 8 Ma (Table 1). Species in the subgenus *Wangenkchetia*, *Paracentris* and *Penthemisia* are associated to *Calceolaria* species (Table 2). A third important member in this system are the bees on the genus *Chalepogenus* (Tapinotaspidini) which are also pollinators of *Calceolaria* flowers (Rasmussen and Olesen 2000). Their particular body size adapted to the exploitation of the pouches formed by the corolla tube is important for the pollination of *Calceolaria* (Rasmussen and Olesen 2000). Relatively young, 8 Ma, but speciose genus, *Calceolaria* seems to have been very benefited by the origin of oil glands, which represented the key innovation in the evolution of this group (Cosacov et al. 2009).

Iridaceae represents one of the most recent family to acquire oil glands for reward oil bee pollinators, only on the last 5 Ma (Table 1). Iridaceae is distributed worldwide, but only in the Neotropics members of the tribe Iridoideae developed oil glands, which evolved multiple times independently (Chauveau et al. 2012). As in the case of Plantaginaceae (Chapter 4), many origins of the oil glands were possible in the evolutionary history of Iridaceae, and apparently not so costly as previously predicted (Renner and Schaefer 2010). There is no evidence of specialization between *Centris* and Iridaceae species, but only

sporadic records of *Centris* (*Paracentris*) species visiting, and pollinating, species of *Cipura*, *Cypella* and *Sysirinchium* (Table 2). Instead Iridaceae oil flowers main pollinators are the bee species in the tribe Tapinotaspidini (A. J. C. Aguiar, unpublished data).

Orchidaceae oil flowers are another case of sporadic visits of *Centris*, but strong association with Tapinotaspidini bees. Oil was gained at least 11 times in the Neotropical Ochidaceae, and several other in the African species, between 12 to 1 Ma ago (Renner and Schaefer 2010). It is difficult to evaluate where and when oil glands was gained in neotropical orchids, because of the lack of a global and robust phylogeny for this hyperdiverse family. It is well-documented that many neotropical Orchidaceae, specially the *Oncidium*-like species, mimicry Malpighiaceae flowers, and could offer or not reward to pollinators (Reis et al. 2007; Carmona-Díaz and García-Franco 2008).

The pollination of the small family Krameriaceae is mainly performed by *Centris* females, which collect only oil on the flowers (Simpson et al. 1977). In our estimates, Krameriaceae is the second oldest family to have gain oil glands. Such as Malpighiaceae flowers, *Krameria* possess epithelial glands on the corolla, which fits with the combs of rigid setae of the four-legged *Centris*, adapted to Malpighiaceae (Neff and Simpson 1981). Being the first oil flower to appear after the Malpighiaceae, certainly involved a kind of mimicry to take advantage from the already existent oil bee pollinators. Indeed *Krameria* flowers, besides being quite different from a malpigh flower (Fig. 2), share similar patterns of pollinator attractives: first, the reward produced in very similar glands, and second the existence of a flag petal which is important to the bee's orientation in the flower (Simpson 1982).

CONCLUSIONS

The three oldest clades of oil producing plants here treated, Malpighiaceae, Plantaginaceae and Krameriaceae, are primarily associated with the oldest groups of oil-collecting bees, *Centris* and *Epicharis*. While the *Epicharis* lineage stuck on the exploitation of Malpighiaceae flowers, *Centris* diversified, occupied new habitats and associated with new plants, developing different morphologies adapted to the new oil sources. The Andean Calceolaria was one of the youngest *Centris'* mutualistic partner to appear, which rapidly diversified in more than 200 species, most of them associated to oil bees. Other oil sources, here dated as originated in the Miocene, Iridaceae and *Nierembergia*, are sporadic partners of *Centris*, but mainly pollinated by the younger group of oil bees, Tapinotaspidini. In driest parts of Neotropical and Nearctic Region, in different time periods, *Centris* freed from the

dependence on oil flowers, and stopped the oil collecting, certainly saving much energy, facilitated probably by the dry condition of the soil, which is the material for these bees's nests.

SUPPLEMENTARY INFORMATION

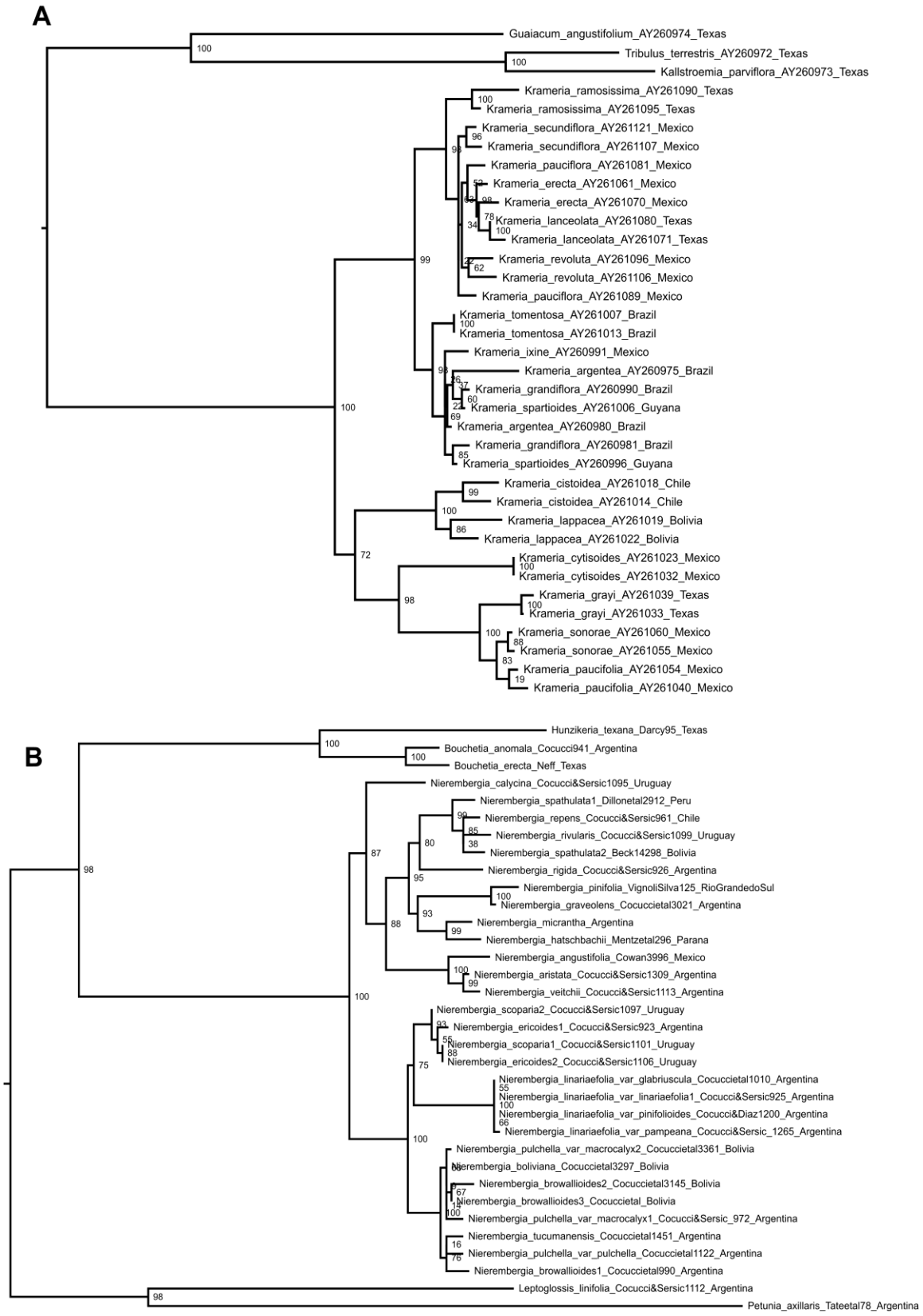

Fig. S1. Maximum Likelihood tree for Krameriaceae and *Nierembergia* (Solanaceae). A. Krameriaceae tree rooted in Zygophyllaceae based on a matrix of 20 taxa and 800 nucleotides. B. *Nierembergia* (Solanaceae) tree rooted in *Petunia* based on a matrix of 35 taxa and 1639 aligned nucleotides. Bootstrap values on nodes.

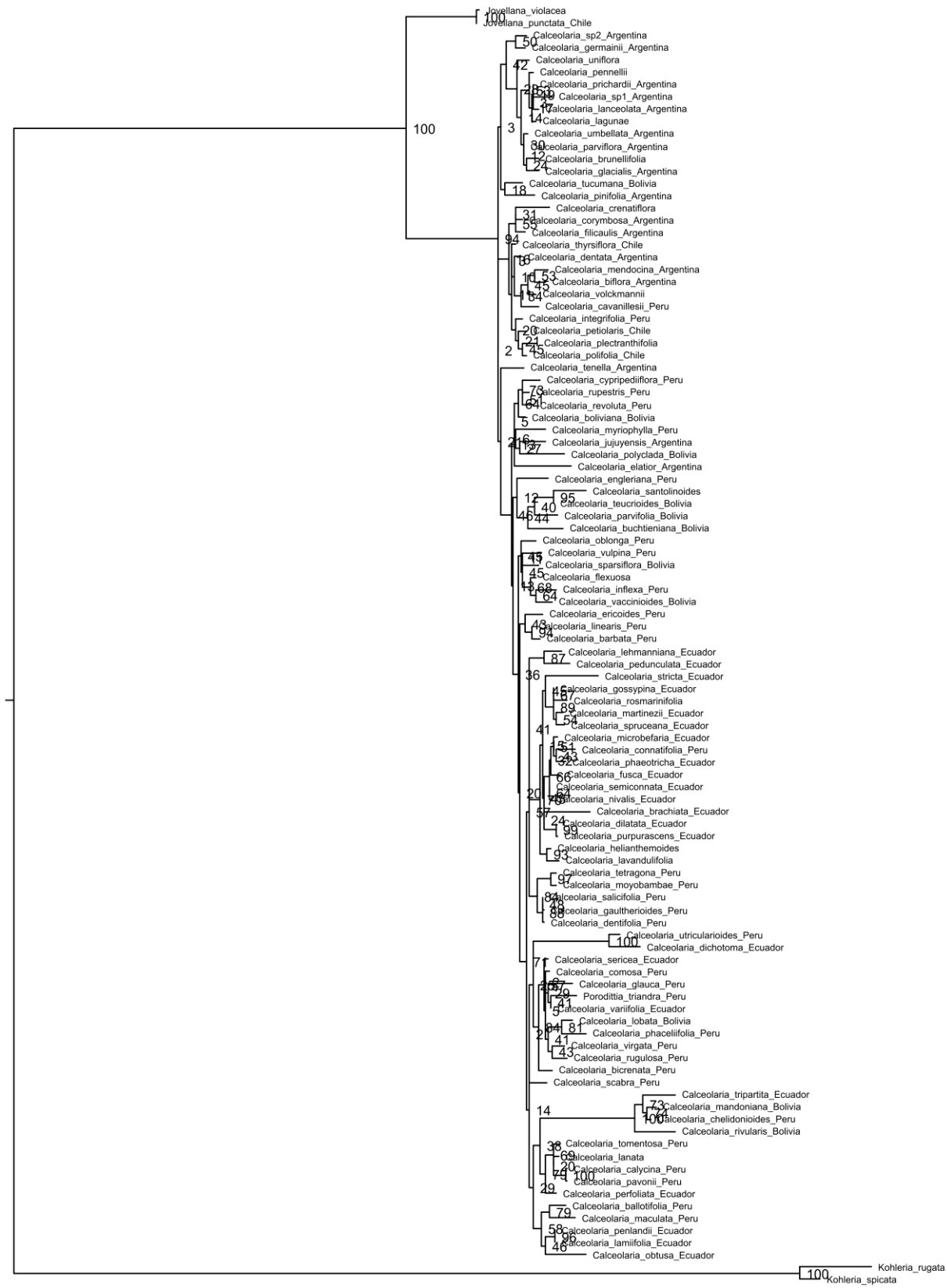

Fig. S2. Maximum Likelihood tree for *Calceolaria* rooted in *Kohleria* (Gesneriaceae) based on a matrix of 103 taxa and 1544 aligned nucleotides. Bootstrap values on nodes.

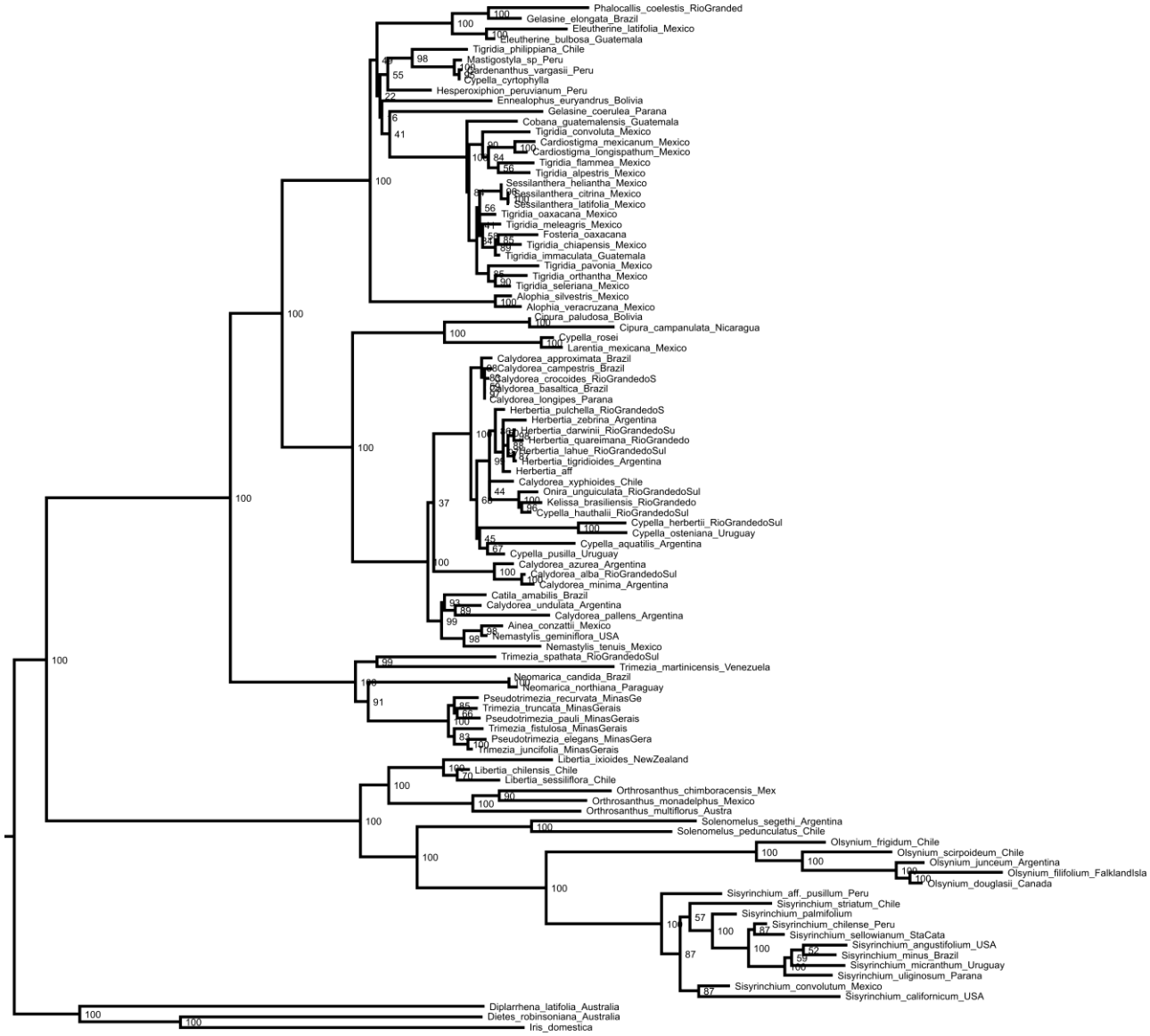

Fig. S3. Maximum Likelihood tree for Sysirinchieae, Trimeziae and Tigridae rooted in *Iris* based on a matrix of 97 taxa and 6000 aligned nucleotides. Bootstrap values on nodes.

Table S1. Bee species and angiosperm families visited for oil-collecting, with relevant references. Genera abreviations: C.: *Centris*, E.: *Epicharis*. Subgenera abreviations: (A.): *Aphemisia*; (C.): *Centris*; (He.): *Hemisiella*; (Ht.): *Heterocentris*; (M.): *Melacentris*; (P.): *Paracentris*; (Pe.): *Penthemisia*; (Pc.) *Ptilocentris*; (Pt.): *Ptilotopus*; (T.): *Trachina*; (W.): *Wagenknechtia*; (X.): *Xanthemisia*; (An.): *Anepicharis*; (Cy.): *Cyphepicharis*; (Ea.): *Epicharana*; (Ep.) *Epicharis*; (Et.): *Epicharitides*; (Eo.): *Epicharoides*; (Ho.): *Hoplepicharis*; (Pr.): *Parepicharis*; (Te.): *Triepicharis*.

Species	**Floral oil producing host families**	**References**
C. (A.) lilacina	?	
C. (A.) mocsary	Malpighiaceae	(Gaglianone 2003; Sigrist and Sazima 2004; Cappellari 2011)
C. (A.) plumipes	?	
C. (*A.*) *agilis*	Malpighiaceae	(Carmona-Díaz and García-Franco 2008)
C. (*A.*) *sericea*	?	
C. (C.) aenea	Malpighiaceae, Plantaginaceae I (*Angelonia*), Krameriaceae	(Vogel 1974; Freitas et al. 1999; Teixeira and Machado 2000; Aguiar 2003; Aguiar and Gaglianone 2003; Gaglianone 2003; Sigrist and Sazima 2004; Gimenes et al. 2007; Martins et al. 2013)
C. (C.) decolorata	Malpighiaceae, Plantaginaceae I (*Angelonia*), Krameriaceae	(Vogel 1974; Teixeira and Machado 2000; Machado 2004; Gimenes and Lobao 2006; Gimenes et al. 2007)
C. (C.) varia	Malpighiaceae, Orchidaceae (*Trichocentrum sitpitatum*)	(Vogel 1974; Teixeira and Machado 2000; Silvera 2002; Gaglianone 2003; Cappellari 2011)
C. (C.) haemorrhoidalis	?	
C. (C.) spilopoda	Malpighiaceae	(Vogel 1974; Teixeira and Machado 2000; Aguiar et al. 2003)
C. (He.) vittata	Malpighiaceae	(Vinson et al. 1997; Aguiar et al. 2003)
C. (He.) tarsata	Malpighiaceae, Plantaginaceae I (*Angelonia*), Krameriaceae	(Freitas et al. 1999; Aguiar et al. 2003; Gaglianone 2003; Machado 2004; Sigrist and Sazima 2004; Gimenes and Lobao 2006; Gimenes et al. 2007; Cappellari 2011)

C. (He.) trigonoides	Malpighiaceae, Krameriaceae, Orchidaceae (*Oncidium sphacelatum*)	(Vogel 1974; Simpson et al. 1977; Aguiar 2003; Gaglianone 2003; Sigrist and Sazima 2004; Damon and Cruz-López 2006; Gimenes and Lobao 2006; Gimenes et al. 2007)
C. (He.) nitida	Malpighiaceae	(Vinson et al. 1997; Downing and Liu 2012)
C. (He.) transversa	?	
C. (Ht.) analis	Malpighiaceae, Plantaginaceae I (*Angelonia*)	(Vogel 1974; Sazima and Sazima 1989; Vinson et al. 1997; Teixeira and Machado 2000; Gaglianone 2003; Machado 2004; Cappellari 2011; Martins et al. 2013)
C. (Ht.) differmis	Malpighiaceae	(Vogel 1974)
C. (Ht.) labrosa	Malpighiaceae	(Sigrist and Sazima 2004)
C. (M.) dimidiata	?	
C. (M.) violacea	Malpighiaceae	(Gaglianone 2003)
C. (M.) dorsata	Malpighiaceae	(Gaglianone 2003; Machado 2004)
C. (M.) rhodoprocta	?	
C. (M.) flavilabris	?	
C. (M.) braccata	?	
C. (M.) conspersa	?	
C. (M.) xanthocnemis	?	
C. (M.) aff. dorsata	?	
C. (*M.*) sp.	?	
C. (*M.*) *gelida*	?	
C. (Pa.) atripes	Krameriaceae	(Simpson et al. 1977)
C. (Pa.) hoffmanseggiae	non oil-collecting	
C. (Pa.) cockerelli	Malpighiaceae, Krameriaceae	(Machado 2004)
C. (Pa.) agameta	?	
C. (Pa.) albiceps	?	
C. (Pa.) anomala	no oil-collecting	
C. (Pa.) nigrocaerulea	?	

C. (Pa.) zacateca	?	
C. (Pa.) hyptidis	Plantaginaceae I (*Angelonia*), Krameriaceae	(Machado et al. 2002; Aguiar et al. 2003; Martins et al. 2013)
C. (Pa.) xanthomelaena	Malpighiaceae, Plantaginaceae (*Angelonia*)	(Vogel and Machado 1991; Aguiar 2003; Aguiar et al. 2003)
C. (Pa.) nigerrima	Plantaginaceae II (*Monttea*), Calceolariaceae	(Vogel 1974; Sérsic and Cocucci 1999)
C. (*Pa.*) *burgdorfii*	Malpighiaceae, Plantaginaceae I (*Angelonia*), Krameriaceae, Iridaceae (*Cipura paradisíaca)*	(Gaglianone 2003; Machado 2004; Cappellari 2011; Martins et al. 2013), Aguiar pers. com.
C. (Pa.) klugii	Malpighiaceae, Plantaginaceae I (*Angelonia*)	(Freitas et al. 1999; Martins et al. 2013)
C. (Pa.) thelyopsis	Plantaginaceae I (*Angelonia*)	(Martins et al. 2013)
C. (Pa.) mexicana	Orchidaceae (*Oncidium sphacelatum*)	(Damon and Salas-Roblero 2007)
C. (Pa.) pallida	?	
C. (Pa.) caesalpinae	?	
C. (Pa.) lanosa	?	
C. (Pa.) tricolor	Malpighiaceae, Plantaginaceae I (*Angelonia*), Plantaginaceae II (*Monttea*); Calceolariaceae, Iridaceae (*Cypella*), Solanaceae	(Vogel 1974; Cocucci 1991; Schlindwein 1995) Aguiar, pers. com.
C. (Pa.) aff. neffi	Calceolariaceae	(Cosacov et al. 2009) (for C. neffi)
C. (Pe.) chilensis	Malpighiaceae, Plantaginaceae II (*Monttea*), Krameriaceae, Calceolariaceae	(Vogel 1974; Simpson et al. 1977; Simpson 1989b; Sérsic and Cocucci 1999; Vivallo et al. 2003)
C. (Pe.) cfr. chilensis	?	
C. (Pe.) tamarugalis	non oil-collecting	
C. (Pe.) bucholzi	Malpighiaceae	(Simpson 1989b)

C. (Pc.) chlorura	?	
C. (Pt.) scopipes	Malpighiaceae	(Gaglianone 2003; Cappellari 2011)
C. (Pt.) decipiens	?	
C. (Pt.) moerens	Malpighiaceae	(Aguiar 2003)
C. (Pt.) sponsa	Malpighiaceae	(Aguiar 2003; Aguiar et al. 2003; Gaglianone 2003)
C. (T.) rupestris	Malpighiaceae	(Cappellari 2011)
C. (T.) similis	Malpighiaceae	(Sazima and Sazima 1989; Gaglianone 2003)
C. (T.) fuscata	Malpighiaceae, Plantaginaceae I (*Angelonia*), Krameriaceae	(Vogel 1974; Vogel and Machado 1991; Vinson et al. 1997; Teixeira and Machado 2000; Aguiar 2003; Aguiar et al. 2003; Gaglianone 2003; Machado 2004; Sigrist and Sazima 2004; Cappellari 2011)
C. (T.) aff. ocellaris	?	
C. (T.) longimana	Malpighiaceae, Orchidaceae (*Trichocentrum sitpitatum*)	(Silvera 2002; Gaglianone 2003)
C. (W.) muralis	non oil collecting	
C. (W.) rhodophtalma	Malpighiaceae, Krameriaceae	(Simpson 1989b)
C. (W.) cineraria	Calceolariaceae, Iridaceae (*Sysinchium*)	(Vogel 1974; Cosacov et al. 2013; Giannini et al. 2013)
C. (X.) bicolor	Malpighiaceae, Plantaginaceae I (*Angelonia*)	(Sazima and Sazima 1989; Gaglianone 2003; Sigrist and Sazima 2004; Cappellari 2011; Martins et al. 2013)
C. (X.) cf. Carolae	?	
C. (X.) lutea Friese, 1899	Malpighiaceae	(Vinson et al. 1997; Gaglianone 2003)
C. sp.	?	
Epicharis (An.) dejeanii	Malpighiaceae	(Gaglianone 2003)
Epicharis (Cy.) morio	?	
Epicharis (Ea.) flava	Malpighiaceae	(Teixeira and Machado 2000; Gaglianone 2003; Sigrist and Sazima 2004; Cappellari 2011)

Epicharis (Ea.) aff. bova	?	
Epicharis (Ea.) elegans	Malpighiaceae	(Gaglianone 2000)
Epicharis (*Ea.*) cf. *lindigi*	?	
Epicharis (Ea.) pygialis	Malpighiaceae	(Gaglianone 2000)
Epicharis (Ep.) bicolor	Malpighiaceae	(Gaglianone 2000, 2003; Teixeira and Machado 2000; Cappellari 2011)
Epicharis (Ep.) umbracullata	Malpighiaceae	(Gaglianone 2000)
Epicharis (Et.) cockerelli	Malpighiaceae	(Gaglianone 2000)
Epicharis (Et.) iheringi	Malpighiaceae	(Gaglianone 2003; Cappellari 2011)
Epicharis (Et.) minima	Malpighiaceae	(Gaglianone 2003)
Epicharis (Et.) luteocincta	Malpighiaceae	(Gaglianone 2000)
Epicharis (Eo.) picta	Malpighiaceae	(Cappellari 2011)
Epicharis (Eo) maculata	Malpighiaceae	(Gaglianone 2000)
Epicharis (Eo.) xanthogastra	Malpighiaceae	(Gaglianone 2003; Cappellari 2011)
Epicharis (Ho.) affinis	Malpighiaceae	(Gaglianone 2000, 2003; Sigrist and Sazima 2004)
Epicharis (Ho.) lunulatta	Malpighiaceae	(Gaglianone 2000)
Epicharis (Pr.) zonata	Malpighiaceae	(Gaglianone 2000)
Epicharis (Te) analis	Malpighiaceae	(Gaglianone 2000, 2003; Sigrist and Sazima 2004; Cappellari 2011)
*Epicharis sp.*1	?	
Epicharis sp. 2	?	

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CAPÍTULO 4

SEVERAL ORIGINS OF FLORAL OIL IN THE ANGELONIEAE, A SOUTHERN HEMISPHERE DISJUNCT CLADE OF PLANTAGINACEAE

SEVERAL ORIGINS OF FLORAL OIL IN THE ANGELONIEAE, A SOUTHERN HEMISPHERE DISJUNCT CLADE OF PLANTAGINACEAE

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- *Premise of the study:* Over the past 75 million years, successive groups of plants have entered the "oil bee pollination niche," meaning that they depend on oil-collecting bees for their pollination. The highly dissimilar numbers of plant species and bee species involved in these mutualisms imply evolutionary host switching, asymmetric mutual dependencies, and uncoupled diversification. Among the clades with the best field data on oil bee behavior is the Angelonieae, which we here investigate to better understand the evolutionary time frame of this pollination syndrome.
- *Methods:* We generated nuclear and plastid data matrices for 56% of the species (plus outgroups) and used Bayesian methods of molecular clock dating, ancestral state reconstruction, and biogeographic inference.
- *Key results:* We found that Angelonieae have of two major clades, *Angelonia* (including *Monopera*) and *Basistemon*, and *Monttea*, *Melosperma*, and *Ourisia*.
- *Conclusions:* Angelonieae originated in dry parts of southern South America in the Middle Eocene, roughly simultaneous with their main oil bee pollinator clade, and switched to oil as a reward four or five times over the past 25 Ma. As predicted in a previous non-clock-dated study, dispersal to Australasia dates to the Miocene/Pliocene.

Key words: Molecular clock; Southern Hemisphere disjunction; Plantaginaceae; oil-offering flowers; parallel evolution

Some 1600 species of plants in 11 families offer oil as a reward to their pollinators. About 1000 of them belong to the Malpighiaceae in which oil is the ancestral reward system, the remainder to the Orchidaceae, which have oil-offering species in many genera, Calceolariaceae (~200, all in *Calceolaria*), Cucurbitaceae (~102 in several genera), Myrsinaceae (~75, all in *Lysimachia*), and just over a dozen small genera of Iridaceae, Krameriaceae, Stilbaceae, Scrophulariaceae, Solanaceae, and Plantaginaceae (Vogel, 1974, 1988; Rasmussen and Olesen, 2000; Machado, 2004; Renner and Schaefer, 2010). How often this reward system has evolved and been lost is unknown, but minimal estimates are 28 origins and 36-40 losses (Renner and Schaefer, 2010) . The oil is used by females of about 365 species of bees from a few tribes and families that use it to provision their offspring or to line their larval cells (Cane et al., 1983; Vogel, 1988; Alves-dos-Santos et al., 2002; Melo and Gaglianone, 2005). Field observations over the past 45 years have shown that these plant/bee interactions are mutually obligatory at the genus or family level, not at the species level (e.g., Rasmussen and Olesen 2000; Machado 2004; Martins et al. 2013), and dated molecular trees have clarified, at least in broad outline, how over the past 75 million years successive groups of plants entered the "oil bee pollination niche" (Renner and Schaefer, 2010). However, the details of the implied switching of bees to new plant species and plant species to new pollinators remain vague, partly for lack of field observations on the bee's foraging breadth, partly for lack of dated and densely sampled phylogenies for the interacting clades.

Among the oil-offering groups with relatively detailed observations on bee behavior on the flowers is the Angelonieae tribe in the Plantaginaceae (Vogel 1974; Simpson et al. 1990; Vogel and Machado 1991; Vogel and Cocucci 1995; Sérsic and Cocucci 1999; Tadey 2011; Martins et al. 2013; Martins and Alves-dos-Santos 2013). This is a small Neotropical group of 68 species in six genera, viz. *Angelonia* (26 species, Barringer, 1981), *Basistemon* (8 spp., Barringer, 1985), *Melosperma* (1 species, (Rossow, 1985), *Monopera* (2 spp., Barringer, 1983), *Monttea* (3 spp., Rossow 1985) and *Ourisia* with 28 species (Albach et al., 2005; Oxelman et al., 2005; Meudt, 2006). The flowers are pollinated by at least 15 species of *Centris* from several subgenera and visited by many further species of Tapinotaspidini (Martins and Alves-dos-Santos 2013 and references therein). The phylogenetic relationships of the bee species pollinating Angelonieae have recently been clarified (Martins et al., 2014). However, understanding the evolutionary relationship between them and their oil sources still

requires a densely sampled phylogeny of the Angelonieae that is geographically explicit and molecular-clock dated. This is crucial since at least two of the six genera, namely *Melosperma* and *Ourisia*, lack oil-producing hair patches (they instead offer nectar to their pollinators) and since one genus, *Basistemon*, is variable for this trait (Barringer, 1985). Depending on the precise phylogenetic relationships among the genera, oil offering could thus have evolved several times in the Angelonieae or it could be ancestral but lost in some species.

To resolve these questions and to shed light on the biogeographic history of the group, we sequenced a nuclear and two plastid DNA markers from 38 of the 68 species representing all genera. Previous phylogenies only sampled a few species of Angelonieae and never included all genera. They could therefore not test whether *Angelonia*, *Basistemon*, and *Monopera*, which share similar saccate corollas (Barringer, 1981, 1983, 1985) or *Melosperma* and *Monttea*, which share curved filaments, similar nectary glands and similar pollen, might form distinct clades (Rossow, 1985; Meudt, 2006). *Angelonia* itself has been divided into three sections based on the length of the corolla tube and the presence of outgrowths on the inner corolla (Barringer 1981; our Fig. 1), characters now known to relate to the size of the pollinator (Martins and Alves-dos-Santos, 2013; Martins et al., 2013), suggesting they could have evolved in parallel as adaptations to particular *Centris* bees differing in body weight and ability to manipulate large or small oil hair fields. The question of the extent of evolutionary liability in oil bee/oil flower co-evolution forms the backdrop to the present study.

MATERIALS AND METHODS

Taxon Sampling–A total of 52 individuals were acquired from several herbaria (Table 1) and represent the three species of *Monttea*, the type species of *Melosperma*, seven of the eight species of *Basistemon* (the missing species, *B. argutus*, is known only from the type collection), the type species of *Monopera*, which has only one other species, and roughly half of all *Angelonia*. We further included 10 of the 28 species of *Ourisia* and as outgroups species of *Bacopa*, *Gratiola*, *Otachantus*, *Scoparia*, *Stemodia* and *Mecardonia* representing the Gratioleae based on Estes and Small (2008) and Schäferhoff et al. (2010).

Molecular Phylogenetics–Total DNA was extracted from 20 mg of leaf tissue using the Nucleospin® Plant II kit (Machery-Nagel, Düren, Germany) and following

the manufacturer's protocol except that after homogenization, the sample was not transferred to a new tube to minimize loss of material. Samples were lysed at 65°C for between one and two hours, the crude lysate was not filtered, and a single pass of elution buffer was utilized to achieve maximum concentration of DNA in the sample. Presence and concentration of DNA in samples was assessed using a NanoDrop® 1000 spectrophotometer (Nanodrop Technologies, Oxfordshire, UK), cleaned with purified water and calibrated with the elution buffer used in the extraction. For recalcitrant samples, we repeated extractions with up to 60 mg of material, using the Invisorb® Spin Plant Mini Kit (Stratec Molecular, Berlin, Germany). We amplified part of the plastid *trn*K intron and *matK* gene, using the primers trnK-2R (ctactccatccgactagtt) and matk8F (cttcgactttcttgtct) (Steele and Vilgalys, 1994) and the *trn*L intron and spacer using the Taberlet et al. (1991) primers, and the nuclear ribosomal DNA internal transcribed spacers 1 and 2, as well as the 5.8 S region, using the newly designed primers 18S-15F (tcgcgacgagaagtccactgaac), 5.8S-434R (tggttcacgggattctgcaa), 5.8S-348F (ggctctcgcatcgatgaaga) and ITS-607R (ctagtcgtgatatccgccgg). The PCR conditions were 94°C for 3 min; followed by 39 cycles of (i) 94°C denaturation for 30 s; (ii) 48-50°C annealing for 1 min, and (iii) 68°C extension for 1 min; and 10 minutes of final elongation. Successful DNA extractions and amplifications were purified, and sequenced using BigDye® and a 3130-4 sequencer (Applied Biosystems, Foster City, CA, USA). Sequences were examined, edited, and constructed into contigs using the Geneious 6.0 software, and all have been deposited in GenBank under the accession numbers presented in Table 1.

The cleaned sequences were automatically aligned using MAFFT (Katoh and Standley 2013) as implemented in Geneious v. 6.1 (Biomatters, 2013) taking into account ITS secondary structure under strategy Q-INS-I (Katoh and Standley, 2013). Alignments were further refined by eye. Phylogenetic analyses were conducted separately for the plastid and nuclear data matrices, and in the absence of statistically supported topological conflict (>70% maximum likelihood bootstrap support), the matrices were concatenated. Maximum likelihood (ML) consensus trees were constructed using RAxML-HPC2 as implemented on the CIPRES server in January 2014 (Miller et al., 2010) with 1000 bootstrap replicates under the GTR + I + Γ model. Bayesian Inference with Markov-chain Monte Carlo (MCMC) was performed in MrBayes 3.2.2 (Ronquist et al., 2012) with the same substitution model and a Markov chain length of 1,000,000 iterations, sampling every 10,000 steps, with a burn-in of

20%. MrBayes runs were assessed for convergence in Tracer 1.5 (Rambaut et al., 2014), and runs with effective sample sizes <150 for any parameter were discarded.

Divergence time estimation–Dating relied on BEAST 1.8.0 (Drummond et al., 2012), using the uncorrelated lognormal relaxed-clock model, a Yule tree speciation model, and the GTR + I + Γ substitution model with empirical base frequencies. MCMC chains were run for 100 million iterations, sampling every 10,000 steps. Convergence and effective sample sizes were assessed in Tracer 1.5, and when effective sample sizes for all parameters were >300, a maximum clade credibility tree was produced in TreeAnnotator 1.8.0 (part of the BEAST package) with a burn-in of 25%. Trees were visualized and manipulated in FigTree 1.4.2 (Rambaut, 2014).

For calibration, we used an ITS rate of 8.34×10^{-3} subs/site/Ma (Primulaceae; Kay et al. 2006) and a plastid rate of 1.3×10^{-3} subs/site/Ma (*trnL-F* region of *Inga*, Richardson et al. 2001). The prior on each rate was a gamma distribution with an initial value of 1.0, and rates were unlinked among the nuclear and plastid partitions.

Ancestral state reconstructions for oil hairs and biogeography–We inferred the evolution of floral oil hair patches, using the states "present" or "absent." Information on the presence/absence of oil hairs came from relevant literature (Vogel, 1974; Barringer, 1981, 1983, 1985; Rossow, 1985; Simpson et al., 1990; Sérsic and Cocucci, 1999; Meudt and Simpson, 2007; Martins and Alves-dos-Santos, 2013). Species of *Angelonia*, *Monopera*, and *Monttea* as well as *Basistemon silvaticus* and *B. spinosus* were coded as "present," the remaining *Basistemon* species as "absent". Barringer (1985) observed a few oil hairs in *B. pulchellus*, and as no data exist on its pollinators, we coded this species once as "absent," once as "present". *Melosperma*, *Ourisia* and outgroups (Gratioleae) were coded as "absent." Ancestral state reconstruction used parsimony and maximum likelihood (MK1 model) in Mesquite v. 2.75 (Madison and Madison, 2011) on a maximum clade credibility chronogram. For the biogeographic reconstruction, species occurrences were compiled from the vouchers included in this study (Table 1) and relevant literature (Barringer, 1981, 1983, 1985; Rossow, 1985; Meudt and Simpson, 2006; Souza and Giulietti, 2009). The ingroup species were assigned to one of the following regions: (A) Caatinga, Cerrado and Chaco area, which are seasonally dry vegetation types (Pennington et al., 2000; Werneck, 2011); (B) Northern Andes; (C) Southern Andes; (D) South American Arid Diagonal, a belt of arid vegetation with <300 mm/year in rainfall that crosses South America from 2°S in the Gulf of Guayaquil to 52°S bounding the Straits of Magellan

(Blisniuk et al., 2005); (E) Central America and Caribbean; (F) Australasia: Australia, Tasmania, New Zealand. The Gratioleae outgroups (*Mecardonia*, with 31 species mostly in South America; *Scoparia*, 2 species in South America; *Gratiola*, 16 species mostly in South America; *Stemodia*, 20 species mostly in South America; *Otacanthus*, ca. 10 species in South America; and *Bacopa*, with 65 species of aquatics, mostly in South America) were coded as South America (G). Reconstruction relied on Bayes-DIVA analysis using Reconstructed Ancestral States in Phylogenies (RASP) v. 2.1 (Yu et al., 2013), also referred to as S-DIVA (Yu et al., 2010). As input, we use 1000 trees from the Bayesian MCMC chain obtained in the BEAST analysis. Results were summarized on the majority rule consensus of the BEAST analysis, which is fully bifurcating.

RESULTS

Species and Genus Relationships–Our *trn*L alignment consisted of 969 sites, the *trn*K-*mat*K alignment of 510 sites, and the ITS alignment of 606 sites. In the absence of any statistically supported (>70 ML bootstrap support) topological conflict between the plastid and nuclear trees, the data were concatenated, yielding a matrix of 2085 sites. The Bayesian and maximum likelihood trees from these data agreed in their topology for all statistically supported nodes (>70% ML bootstrap support or >99% posterior probability). *Ourisia* is sister to *Melosperma* and *Monttea* (Fig. 1), and a clade of the other three genera, *Angelonia, Basistemon,* and *Monopera*, also has high support, with *M. micrantha*, the type species of the genus, deeply nested within *Angelonia*.

Divergence Times and Biogeography–The chronogram obtained from the concatenated matrix (Fig. 2) yielded a root age, i.e., the split between the Angelonieae and Gratioleae, in the Middle Eocene at 46 (34-61) Ma (95% highest posterior density interval), a crown age of the Angelonieae in the Uppermost Eocene at 35 (26-47) Ma, a crown age of the *Angelonia/Basistemon* clade in the Upper Oligocene at 25 (18-34) Ma, and a crown age of the *Monttea*/*Ourisia* clade in the Upper Oligocene at 30 (20-40) Ma. We take up the issue of cross validation of these estimates in the *Discussion*. The biogeographic reconstruction (Fig. 2) gave ambiguous results for the Angelonieae, which originated either in seasonally dry South American biomes or the Southern Andes. *Angelonia* appears to have initially diversified in the Caatinga, Cerrado and Chaco area, with some northwards expansion to Central America and the Caribbean, in our sampling represented by the Cuban species *A. pilosella*. The *Monttea/Ourisia* clade

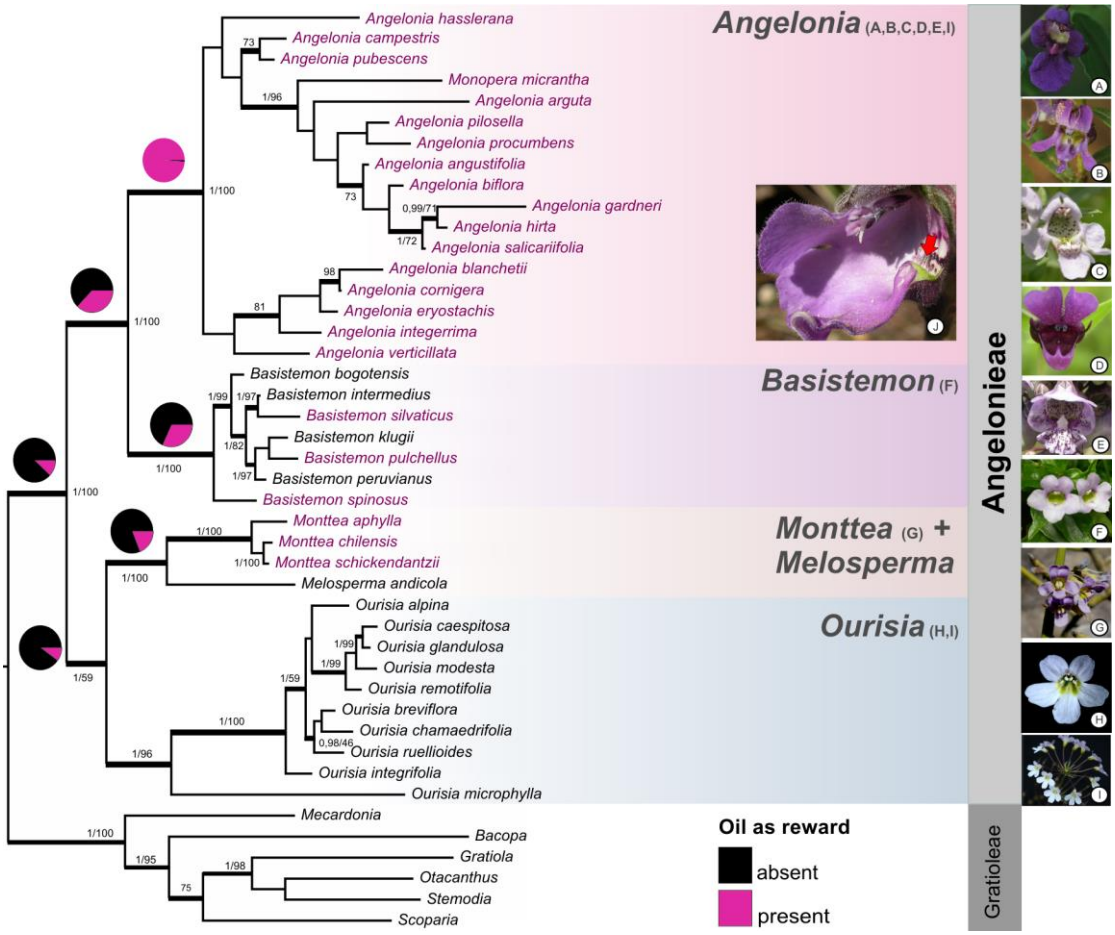

Figure 1. Bayesian consensus tree resulting from the analysis of the combined nuclear and plastid data (44 species and 2085 aligned nucleotides), rooted between Angeloniae Gratioleae. Bayesian posterior probability (BPP) values ≥98% and maximum likelihood bootstrap support (BS) values ≥70% are shown at nodes. Thickened branches are supported either by ≥98% BPP and/or ≥70% BS. Pie charts at internal nodes indicate the ancestral states of the production of floral oil inferred under likelihood optimization on the maximum clade credibility tree with the color indicating the most plausible state: absent (black) or present (purple). Purple branches indicate the presence of floral oil. Photos on the right show the floral morphology of the following species: A. *Angelonia campestris*, B. *A. arguta*, C. *A. salicariifolia*, D. *A. cornigera*, E. *A. integerrima*, F. *Basistemon silvaticus*, G. *Monttea aphylla*, H. and I. *Ourisia macrophylla*. J. *Angelonia eryostachis* flower cut to show the green oil hair carpet (red arrow); corolla tube length 3 cm. Photos: A-B, A. Aguiar, C- F and I, A. Martins, G, M. Taniguchi; H, P. Garnock-Jones.

apparently originated in the Southern Andes, with two independent dispersal events from southern South America (probably Chile) to Tasmania/New Zealand at 6 (3-10) Ma and 3 (1-5) Ma ago (Fig. 2).

Ancestral state reconstruction of the floral rewards–Figure 1 (pie diagrams at nodes) shows the inferred evolution of floral oil as a reward in the Angelonieae obtained with the maximum likelihood reconstruction, which agreed with the parsimony-based

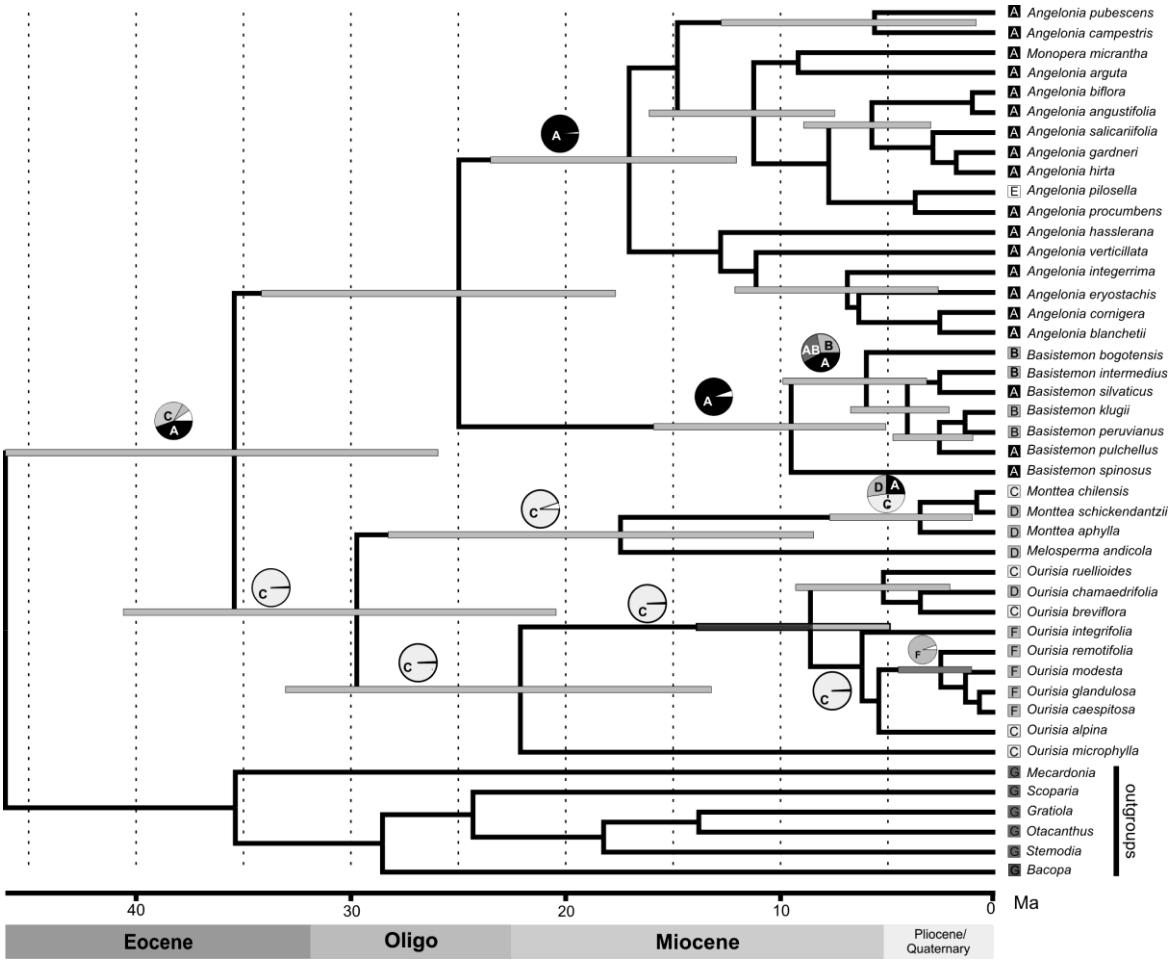

Figure 2. Chronogram for Angelonieae rooted on Gratioleae obtained under a Bayesian relaxed clock model and the same as used in Fig. 1. Bars indicating 95% probability are shown for all well-supported nodes (Fig. 1). Pie charts at internal nodes indicate the probabilities for each alternative ancestral area derived from Bayesian ancestral reconstruction over 1000 dated trees. Squares indicate the present geographic distribution of the species, with the areas being: A. South American open vegetation biomes; B. Northern Andes; C. Southern Andes; D. South American Arid Diagonal; E. Central America and Caribbean; F. Australasia; G. South America.

reconstructions. The most plausible ancestral condition is the absence of floral oil and five independent gains, once in the common ancestor of *Angelonia*, once in the common ancestor of *Monttea* and three times in *Basistemon,* where *B. silvaticus* and *B. spinosus* produce oil, and *B. pulchellus* has at least few oil hairs. When this species was coded as oil producing (as shown in Fig. 1), the ancestral state in *Basistemon* was "oil present" with 34% probability, when it was coded as "oil absent," that probability dropped to 32%. Unfortunately, a third oil-producing species, *B. argutus,* could not be sequenced. The alternative scenario, with the ancestral state of the Angelonieae being "oil hairs present" would require one gain in the ancestor, one loss in the common ancestor of *Melosperma, Ourisia, Monttea,* followed by a regain in *Monttea* and several losses in *Basistemon,* and is thus less parsimonious.

DISCUSSION

Species and Genus Relationships–Morphological and taxonomic work by Barringer (1985) suggested that *Angelonia, Monopera,* and *Basistemon* might share a common ancestor because of their similar saccate corollas and capitate stigmas. This inference is supported by the molecular phylogenetic results presented here. Likewise, *Monttea* and *Melosperma* were seen as closely related based on curved filaments and their similar nectary scales (Rossow, 1985; Sérsic and Cocucci, 1999). However, their sister group relationship to *Ourisia* was so far unknown, probably due to insufficient taxon sampling as suggested by Meudt (2006). O*urisia* has the same nectary scales as *Monttea* and *Melosperma,* but like *Melosperma* lacks oil-producing hairs in its flowers, while the three species of *Monttea* have such hairs and floral oils. We discuss the implications of this for the evolution of the oil-flower syndrome below.

The nesting of *Monopera* in *Angelonia* agrees with the original placement of the two species of this genus. The type species of *Monopera* was described as *Angelonia micrantha* (Bentham, 1846), and the second species, *M. perennis* (Chodat & Hassl.) Barringer, was originally *A. micrantha* Benth. var. *perennis* Chodat & Hassl. The only nomenclatural change needed now is the return these entities to *Angelonia*. Barringer's (1983) reason for separating *Monopera* was the unisaccate corolla, which he thought was quite different from the bisaccate corolla typical of *Angelonia,* while the molecular tree implies that this is a derived form of corolla. Our species sampling within *Angelonia* is still too sparse to fully assess the three sections of Barringer (1981).

Cross Validation of Divergences Times and Southern Hemisphere Long Distance Dispersal–Our dating analysis yielded a crown age of the Angelonieae in the Uppermost Eocene at 35 (26-47) Ma, slightly younger than the age for the Plantaginaceae of 46 (36-57) Ma inferred by Bell et al. (2010), who only included five representatives of this family of ca. 90 genera. The *Centris* clade of oil bees that is most closely associated with the oil-offering Angelonieae is 44 (31-55) Ma old (Martins et al., 2014), which would place the diversification of these bees at roughly at the same time as the divergence between the two main clades within the Angelonieae. A recent phylogeographic study of *Monttea chilensis* that used *M. schickendantzii* to root their analyses, dated the split separating these two species to 5.6 (2–6) Ma, with a published plastid substitution rate, or 11 (5–16) Ma with a nuclear substitution rate (Baranzelli et al. 2014), while we inferred an age for this split of 0.8 (0.1-2.6) Ma. This difference is probably due to a combination of the slightly faster plastid and nuclear substitution rates used here compared to their study and their dense population sampling within *Monttea* (they have 13 individuals in their plastid tree and 28 in the nuclear tree). The early diversification of the Angelonieae clades occurred largely in the dry parts of Chile and the western coast of South America.

A molecular phylogenetic study by Meudt and Simpson (2006) already showed that *Ourisia*, which has one species in Tasmania and Australia, 12 in New Zealand, and 15 in the South American Andes, originated in southern South America from where it may have dispersed to Australia/Tasmania and New Zealand. Their study refrained from molecular clock analysis, and their trees were rooted on more distant Plantaginaceae, instead of the true sister clade *Monttea*/*Melosperma* (our Figs. 1 and 2), which is native to Chile and Argentina just as the South American species of *Ourisia.* Our study confirms their inference of two long distance dispersal events, perhaps near the Miocene/Pliocene boundary. Similarly recent transoceanic dispersals have been inferred in the legume genus *Sophora* (Hurr et al., 1999, 2-5 Ma), the Atherospermataceae *Laurelia* (Renner et al., 2000, 50-30 Ma), the grass *Oreobolus* (Chacón et al., 2006, 3-4 Ma) and the Alstroemeriaceae genus *Luzuriaga* (Chacón et al., 2012, 3 Ma).

Origins of the Oil-Offering Syndrome in the Angelonieae–Plantaginaceae often have glandular hairs on their corollas, which is the key morphological pre-adaptation facilitating the transition from nectar to oil as a reward (Fig. 1 photo I). If female oil bees that visited *Ourisia*-like flowers for nectar or pollen would occasionally try to

exploit the glandular hair patches on the inner corolla and pollinate the flowers in the process, this would have set up the selective conditions for switching from nectar to oil as the main reward. This scenario is made more plausible by the observation that oil bees sometimes harvest glandular exudates even from non-floral plant hairs (Melo and Gaglianone, 2005). The flowers of *Monttea*, like those of its closest relatives *Melosperma* and *Ourisia*, still have nectary disks (Meudt 2006: 19), while at the same time possessing oil hair patches and being pollinated by medium-sized oil-collecting *Centris* (Simpson et al., 1990; Tadey, 2011). Bees from the particular subgenera of *Centris* (Centridini) and from the genus *Caenonomada* (Tapinotaspidini) interacting with Angelonieae have pads of soft hairs on their forelegs adapted to absorbing the oil from the hairs, which are <1 mm high (Simpson et al., 1990; Martins et al., 2013). While exploiting the oil glands, the bees stabilize themselves by holding onto the filaments with their mandibles and their head then comes into contact with the stigma (Martins et al., 2013: Photo 5). Relatively long glandular hairs on the inner flower lip also characterize the genus *Mecardonia* (15 species) in the sister clade of the Angelonieae, the Gratioleae (Cappellari et al., 2009). In that case, however, it is male oil bees that collect the oil; there is no nectar.

The repeated gain of oil hairs in *Basistemon* is of particular note. At least three species of this genus (of eight species total) produce oil, and one of these could not be sequenced. A fourth species, *B. pulchellus*, has only very few oil hairs, and we therefore coded it once as oil producing, once as not oil producing (Fig. 1 shows it coded as oil producing). Even with the missing oil-producing species included, the ancestral state would probably remain ambiguous, given that four of eight species lack oil. Field work is required to better understand why some *Basistemon* returned to relying only on pollen as a reward, instead of also offering oil or nectar (Barringer, 1985).

Conclusions–Our results reveal that oil as a pollinator reward evolved four or five times within the Angelonieae, a clade that may date to the Eocene, matching the recently inferred crown age of the *Centris* oil bee clade that is most closely associated with (Martins et al., 2014). We also found that the species and subspecies of *A. micrantha* that were separated and ranked as the genus *Monopera* should be returned to *Angelonia* and that *Ourisia* is sister to *Monttea* and *Melosperma*, with which it shares characteristic nectary scales. The two long distance dispersal events from Chile to Australasia in *Ourisia* appear to have occurred about 3 to 6 to Ma ago, adding yet another example of Pliocene transoceanic dispersal among the continents of the

Southern Hemisphere.

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Table 1. List of species used in this study with author names and collection data and GenBank accession numbers.

a) Newly sequenced species				**Plastid**		**Nuclear**
DNA code	**Species**	**Voucher (Herbarium)**	**Geographic collecting data**	**trnk-matk**	**trnl-F**	**ITS**
AM133	*Angelonia arguta* Benth.	E. Duarte 421 (M)	Brazil: Bahia, 10km. de Tucano, entre Tucano e Euclides da Cunha. E. Duarte 421 e A. Castellanos. 15 Jul. 1964	KM281707	-	KM281689
AM180	*Angelonia arguta* Benth.	A. Aguiar (UB)	Brazil, Goiás, Flores de Goiás, Margem do Rio Macaquinho, 19.Jan.2013		KM281676	
AM153	*Angelonia biflora* Benth.	A. C. Martins 200 (M)	Cultivated in the Munich Botanical Garden (acc. 13/0882) from seeds originally collected in Brazil and provided by the Botanical Garden Plantarum, Av. Brasil 2000, Nova Odessa, São Paulo, Brazil. 24 Oct 2013.	KM281708	KM281677	KM281690
AM136	*Angelonia blanchetii* Benth.	R. Harley 16787 (M)	Brazil. 8 km N.W. Of Lagoinha (5.5 km. S.W. Of Delfino) on the road to Minas do Mimoso. Caatinga/Cerrado, frequently burn and cut over. Alt. Ca 850 m. Aprox. 41°17'W, 10° 24'S. R M Harley No16787	KM281709	KM281678	-
AM131	*Angelonia campestris* Nees & Mart.	R. Harley 16756 (M)	Brazil. 3km. N.W. Of Lagoinha (5.5 km. S.W. of Delfino) on the side road to Minas do Mimoso. Cut over woodland, and by cultivation near river. Alt.ca. 640 m. Aprox. 41°16'W, 10°27'S, 5 Mar. 1974 R.M. Harley No. 16756	KM281710	KM281679	-
AM134	*Angelonia cornigera* Hook.	R. Harley 16302 (M)	Brazil. 64 km. North of Senhor do Bonfim on the BA 130 highway to Juazeiro. Caatinga and disturbed ground on sandy soil with standing water and damp ground. Alt ca. 400m Aprox. 40°15'W, 9°55'S, 25th. Feb. 1974, R.M. Harley No. 16302	KM281711	KM281680	KM281691
AM177	*Angelonia eryostachis* Benth.	A. C. Martins (ESA)	Brazil, Minas Gerais, Parque Estadual do Rio Preto, 18°12'S, 43°19'W, 1500 m	KM281712	KM281681	KM281692

AM184	*Angelonia gardneri* Hook	S. Beck 27908 (LPB)	Bolivia, Santa Cruz, 6 Jan 2003	KM281713	KM371016	KM281693
AM147	*Angelonia hasslerana* Chodat	E. Zardini 33730 (MO)	Paraguay, Central, 18 Nov. 1992	KM281714	-	KM281694
AM144	*Angelonia hirta* Cham.	Rodrigues 30 (MO)	Brazil, Pernambuco, 4 Apr.1995	KM281715	-	KM281695
AM178	*Angelonia integerrima* Spreng.	A. C. Martins (ESA)	Brazil, Rio Grande do Sul, Porto Alegre, Parque Natural Morro do Osso, 30°07'S, 51°14'W, 143 m	KM371019	KM371014	-
AM137	*Angelonia pilosella* J. Kickx f.	Curtis (M)	Near Nueva Gerona, Isla de Pinos, West Indian, 26 Apr. 1904	-	KM281682	-
AM181	*Angelonia pilosella* Kickx f.	W. Greuter & Hankin 26861 (B)	Prov. Pinar del Rio, municipio Consolación del Sur: cerca del restaurante Barrigona, 3km al OSO de la salida de la autopista para Herradura (caudrícula 29-24).	KM281716	-	-
AM142	*Angelonia procumbens* Nees & Mart.	Paixao 87 (MO)	Brazil, Bahia, 18 Nov. 1998	KM281717	KM281683	KM281696
AM179	*Angelonia salicariifolia* Bonpl.	A. C. Martins (ESA)	Brazil, Mato Grosso do Sul, Porto Murtinho, 21°37'S, 57°49'W, 90 m	KM281718	KM371015	KM281697
AM175	*Angelonia verticillata* Philcox	P. von Lützelburg 176 (M)	Brasilia (sic. Brasil): Bahia. Serra das Almas. 1600m. Carrasco. 1200 m 1914.	KM371017	KM281684	KM281698
AM150	*Basistemon bogotensis* Turcz.	H. van der Werff 7448 (MO)	Venezuela, Falcón	KM281719	-	KM281699
AM187	*Basistemon intermedius* Edwin	Seidel & Schulte 2184 (LPB)	Bolivia, La Paz, Sud Yungas, 700 m	KM281720	-	KM281700
AM135	*Basistemon peruvianus* Benth. ex B.D. Jacks.	G. Hatschbach 74234 (M)	Projeto Guaicurus, início da rodovia para Mouraria do Sul (Mun. Bonito). Mato Grosso do Sul. G. Hatschbach, M. Hatschbach & J.M. Silva 74234. 15 Nov. 2002	KM371018	KM281685	KM281701
AM188	*Basistemon pulchellus* (S. Moore) Barringer	J. Wood & Soto 25300 (LPB)	Bolivia, Beni or Pando	KM281721	KM281686	KM281702
AM186	*Basistemon spinosus* (Chodat) Moldenke	J. Wood & Mendoza 21479 (LPB)	Bolivia, Santa Cruz, Vallegrande (18°43'28"S 064°18'47"W, 950 masl) 25 Jan. 2005	KM281722	KM371013	-
AM191	*Basistemon spinosus* (Chodat) Moldenke	M. Dematteis 1972 (MBM)	Bolivia, Dep. Santa Cruz, Prov. Cordillera. 34 km N de Boyuibe,	-	-	KM281703

				20°15'56"S, 63°29'50"W, 896 m.s.m. 29/03/2006			
AM139	*Monopera micrantha* (Benth.) Barringer	(M)		Goncalo (?), Apr. 1916	KM281723	-	KM281704

b) Ingroup Sérsic et al. Sequences

	Monttea aphylla (Miers) Benth. & Hook. F.	AAC (CORD)	4463	Argentina, Neuquen, Confluencia Mol 11, -38.9574 -69.0742	-	KM281687	KM281705
	Monttea schickendantzii Griseb.	AAC (CORD)	4516	Argentina, Catamarca, Barranca Larga, -26.9713 -66.7383	-	KM281688	KM281706

c) Ingroup and Outgroup GenBank accessions

				Plastid		Nuclear
Species	**Voucher**		**Collecting data**	**trnk/matk**	**trnL-F**	**ITS**
Angelonia angustifolia Benth.	McGregor 16461 (TEX)		MEXICO (Puebla)	AY492127.1	-	-
Angelonia pubescens Benth.	C. W. de Pamphilis 94.03 (PAC)			-	AJ608618.1	-
Bacopa monnieri (L.) Edwall	Turner & Turner 97-527, (TEX)		USA: TX, Crockett County	AY492129	AY492170	AY492095
Basistemon klugii Barringer	T. Plowman & al. 11387 (GB)		Mariscal Caceres 09°09'S 076°27'W	AJ608616.1	AJ608616.1	-
Basistemon silvaticus (Herzog) Baehni & J.F. Macbr.	M. Nee 38032 (TEX)		BOLIVIA. Santa Cruz	AY492130.1	GBAY492171.1	AY492096
Gratiola officinalis L.	D. Albach 490		BULGARIA: Rhodopes mountains	AY492143	-	AY492106
Gratiola pillosa Michx.				--	AJ608591	
Mecardonia procumbens (Mill.) Small	Denny & R. Harvey 449, (TEX)		USA:TX, Bandera County	AY492152	AY492184	AY492111
Melosperma andicola Benth.	Arroyo and Humaña 206607 (TEX)		CHILE: Región Metropolitana	AY492185.1	AY492153	AY492112
Monttea chilensis Gay	M. Dillon 8132		Chile: II Region de Antofagasta	AY492187	AY492155	AY492114
Otacanthus sp.	D. Albach ex BG Bonn, (WU)		BG Bonn (collected in Brazil)	AY492188	AY492156	AY492115
Ourisia alpina Poeppig & Endlicher	H. Meudt et al. 40		CHILE: IX Región de la Araucanía, Sierra Nevada	AY613154	-	AY613110

Ourisia breviflora Bentham in DC	H. Meudt & López 058	ARGENTINA: Prov. Tierra del Fuego, Paso Garibaldi.	AY613155	-	AY613111
Ourisia caespitosa J.D. Hooker	H. Meudt & López 075	NEW ZEALAND: North Island, Mt. Ruapehu.	AY613159	JN246141	AY613115
Ourisia chamaedrifolia Benth. in DC.	H. Meudt & López 052	PERU: Depto. Cusco, Prov. Paucartambo.	AY613160	-	AY613116
Ourisia glandulosa J.D. Hooker	H. Meudt & López 090	NEW ZEALAND: South Island, Otago, the Remarkables	AY613168	JN246146	AY613124
Ourisia integrifolia R. Brown	H. Meudt & López 066	AUSTRALIA: Tasmania, Hartz Mountains	AY613171	-	AY613127
Ourisia microphylla Poepp. & Endl.	H. Meudt & López 036	CHILE. VII Región del Maule, Cañon del Río Maule	AY492157	AY492189	AY613136
Ourisia modesta Diels	H. Meudt & López 079	cult ex NEW ZEALAND: South Island, Nelson Hope Range	AY613181	-	AY613137
Ourisia remotifolia Arroyo	H. Meudt & López 094	NEW ZEALAND: South Island, Southland, Gertrude Saddle	AY613187	JN246158	AY613143
Ourisia ruellioides Kuntze	H. Meudt & López 013	Argentina: Prov. Santa Cruz, Perito Moreno	AY613190	AJ608579	AY613144
Scoparia dulcis L.	E. Fischer 10254 (Bonn)		FN773556	FN794072	AY492119
Stemodia durantifolia (L.) Sw.	Reina et al. 98-198, (TEX)	Mexico: Sonora	AY492164	-	AY492120
Stemodia glabra Oerst.	B. Nordenstam & A. Anderberg 967 (S)		-	AJ608566	-

Note. Herbarium acronyms: (M) Botanische Staatssammlung München, Germany, (UB): Universidade de Brasilia, Brazil; (LPB) Herbario Nacional de Bolivia, Universidad Mayor de San Andrés, Bolivia; (ESA) Universidade de São Paulo, Brazil ; (MO) Missouri Botanical Garden, United States; (B) Botanischer Garten und Botanisches Museum Berlin-Dahlem, Zentraleinrichtung der Freien Universität Berlin, Germany; (CORD) Universidad Nacional de Córdoba, Argentina; (TEX) University of Texas at Austin, United States; (WU) Universität Wien, Austria; (S) Swedish Museum of Natural History, Sweden;